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Progressive Changes in Renal Phosphate and Calcium Excretion in Rats Following Parathyroidectomy or Parathyroid Administration.* (21353)

ROY V. TALMAGE AND F. W. KRAINTZ.

From the Biology Department, The Rice Institute, Houston, Texas.

In spite of the ease with which it is possible to show changes in phosphate excretion following parathyroid extract administration or parathyroidectomy, the role played by the kidney in overall parathyroid control of phosphate and calcium metabolism is not yet fully understood. One difficulty is the problem of distinguishing excretion changes due to direct renal function from those due to changes in rate of entry or withdrawal of phosphate and calcium between serum and extra-vascular sources. A second problem is the possibility that parathyroid extracts used in physiological studies, since they contain 2 or more protein components, may produce non-physiological actions when administered to animals. The effect of parathyroid extract on renal clearance has been difficult to interpret and has given rise to conflicting results. This has probably resulted from the 2 factors cited above coupled with the point recently brought out by Barrter (1) that renal clearance studies are of little value during the period in which the serum levels of the ions under study are changing. Because of recent emphasis on the extra-renal influences of parathyroids(2-5), it has been suggested that the increase in phosphate excretion produced by parathyroid extract administration might be an artefact(6). More conservatively, others(7,8) have suggested that the 2 functions, urinary phosphate excretion activity and calcium mobilizing activity, of parathyroid extract may be independent of each other. On the other hand, it has been shown recently(9) that the generally accepted conclusion that parathyroids directly influence kidney excretion of phosphate is a valid one

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which cannot be discarded. In the past it has been considered that changes in renal excretion of calcium which follow changes in parathyroid hormone titer in the body were the result of a changed serum level of this ion. The kidney, therefore, has been considered to play only a passive role in this regard. The first indication that kidneys might also be involved directly in these calcium excretion changes was the recent work of Talmage *et al.*(10) in which the primary emphasis was on calcium turnover in bone. Because of its importance, this point has been investigated further, and the studies reported here indicate a direct renal involvement in the influence of the parathyroids on calcium metabolism.

These studies are also concerned with progressive changes in renal excretion of both calcium and phosphate during the first 27 hours following either parathyroid removal or the first injection of parathyroid extract into normal rats. Earlier work(9) has shown that a major drop in phosphate excretion occurs immediately after parathyroidectomy. This study provides information indicating that this as well as the calcium changes are transitory.

Material and methods. Approximately 150 male, Sprague-Dawley rats weighing between 180 and 225 g were used. Parathyroids were removed by the method of Richter and Birmingham(11). Parathyroid extract† was administered subcutaneously in multiple doses in amounts as indicated in individual experiments. Two series of experiments were run. In the preliminary series parathyroidectomy or the first injection of parathyroid extract, was followed one hour later by administration of the radioisotope (Ca^{45} or P^{32}), and a water dose of 5% of body weight. The animal was then placed in a metabolism cage and 3 successive collections of urine were made covering hours 2-4, 5-12, and 13-24. In place of free access to water, each animal was given an additional 5 ml of water during each of the latter 2 periods. The animals were killed at the conclusion of 24 hour period and blood obtained for serum calcium and phosphate analyses. In the second series of experiments, the animals were divided into 2 groups. The first group was treated as described above except that the experiment was concluded after the first urine collection period and blood was obtained for serum analyses. In some of these experiments, urine output for the second and third hours was collected separately and in others the animals were killed at end of second hour to obtain blood for analysis. The second group was maintained in stock cages for 24 hours after start of the experiment. Multiple doses of parathyroid extract were administered during this period. At the conclusion of the 24 hour period, each animal was given the usual dose of radioactivity and water and placed in a metabolism cage. Urine was collected between the 24th and 27th hours following which the animal was killed to obtain blood for analysis. To establish that operative procedures did not in themselves produce any of the results, sham operations were done in several experiments. As soon as it was thoroughly established that these sham-operated controls responded identically to normal animals, this practice was discontinued. The radioactive substance was administered in doses of 7 to 10 μc, but kept constant for each experiment. A minimum of 4 control animals was included in each experiment. Determinations of radioactivity were made by standard procedure using a G.M. Counter. In the case of Ca^{45}, appropriate self-absorption corrections were made. Calcium determinations were made by the method of Clark and Collip (12); phosphate determinations by the method described by LePage(13).

Results given below deal with changes in phosphate and calcium excretion during the first 27 hours after either parathyroidectomy or the first administration of parathyroid extract. Since these excretion changes spectacularly reversed themselves during this period, it was important to determine that the animals were not recovering physiologically from the experimental treatment. In Table I are tabulated serum values for the 2nd, 4th, and 27th hours. The purpose of this tabulation is to show that the animals became progressively more hypoparathyroid, or more hyperparathyroid, throughout the experimental period as a result of treatment and progressive changes in

† Lilly's Parathyroid Extract.

TABLE I. Comparison of Normal Phosphate and Calcium Serum Values to Experimental at 4 and 27 Hours after Start of Experimental Period.

	Treatment	2 hr	4 hr	27 hr
Serum phosphate, mg P/100 ml	Parathyroidectomized	9.6 ± .25 (16)	9.8 ± .31 (27)	13.7 ±2.3 (28)
	Normal	9.2 ± .24 (30)	9.2 ± .24 (30)	9.2 ± .24 (30)
	Parathyroid extract	—	8.5 ± .27 (22)	7.9 ± .43 (6)
Serum calcium, mg Ca/100 ml	Parathyroidectomized	10.4 ± .19 (16)	9.0 ± .14 (30)	6.3 ± .17 (28)
	Normal	11.1 ± .14 (43)	11.1 ± .14 (43)	11.1 ± .14 (43)
	Parathyroid extract	—	11.3 ± .15 (22)	13.4 ± .29 (6)

Numbers in parentheses are No. of animals in group. Values are given with S.E.

urinary excretions described could not, therefore, be ascribed to the return of the animal to a normal physiological state.

Continuous urine collection: Preliminary experiments were run with small groups of rats in which the urine was collected continuously during the experiment as described above. These experiments indicated that following removal of the parathyroids there was an immediate and marked drop in phosphate excretion and a corresponding though not as marked rise in urinary calcium. The differential in the excretory rates for these 2 ions, between parathyroidectomized and normal animals, persisted at least through the first 12 hours, but during the second 12 hour period both urinary calcium and phosphate appeared to return to normal. The second series of experiments described above, provided statistical comparison between these early excretory rates and corresponding serum values, and those which existed 27 hours after beginning of the experimental period. These experiments are reported below in 3 sections.

Urinary phosphate excretion, hours 1-4. The immediate drop in phosphate excretion, following parathyroidectomy, has been discussed previously(9). Additional data in this regard are summarized in Table II. Also summarized are the effects of parathyroid extract on phosphate excretion during the first 4 hours of administration. It will be noted that the increase in phosphate excretion produced by the extract is statistically higher than the normal, but was much less marked than the drop noted after parathyroidectomy.

Urinary calcium excretion, hours 1-4. Early changes in renal excretion of calcium following parathyroidectomy and following parathyroid extract administration are summarized in Table II. Due to the greater cost of essentially carrier-free Ca^{45}, some experiments were done with Ca^{45} which contained significant amounts of carrier calcium. While up to 0.5 mg of carrier calcium could not be shown to have any significant effect on total calcium excreted per hour, it did markedly affect the radiocalcium excreted, and increased the difference between the experimental and control groups. This phenomenon cannot as yet be

TABLE II. Comparison of Calcium and Phosphate Excretion at 4 Hr after Start of Experimental Period (Urine Collection Period—Hr 2-4).

	Parathyroidectomized	Normal	PTH*
Ca, mg/hr	(25) .174± .019	(39) .037± .002	(18) .026± .001
Total Ca^{45}†	(12)2024 ± 182	(22)1000 ±101	(10) 480 ± 71
Total Ca^{45}, .5 mg Ca‡	(12)8520 ±1400	(17)1000 ±185	(6) 153 ± 25
P, mg/hr	(19) .015± .002	(40) .40 ± .046	(22) .68 ± .07
Total P^{32}†	(34) 23 ± 6	(32)1000 ± 62	(6)1654 ±248
	(Following 2 items, value for second hr only.)		
Ca, mg/hr	(28) .178± .024	(12) .055± .025	

Numbers in parentheses are No. of animals in group. Values are given with S.E.

* These animals received 100 i.u. parathyroid extract in 4 injections during first 3 hr of experimental period.

† For comparative purposes, radioactivity excreted by control animals was set at 1000 for 3 hr collection period, and values for experimentals adjusted to these standards.

‡ These animals received .5 mg carrier calcium with radioactivity.

TABLE III. Comparison of Calcium and Phosphate Excretion at 27 Hours after the Start of Experimental Period (Urine Collection Period—Hours 25-27).

	Parathyroidectomized	Normal	PTH*
Ca, mg/hr	(19) .031± .003	(20) .040± .004	(6) .056± .004
Total Ca^{45}†	(10) 202 ± 60	(8)1000 ±200	(5)2040 ±670
$PO_4^{=}$, mg P/hr	(20) .58 ± .072	(20) .38 ± .067	(6) .94 ± .11
Total P^{32}†	(14)1776 ±1170	(13)1000 ±117	—

Numbers in parentheses are No. of animals in group. Values are given with S.E.

* These animals received 155 units parathyroid extract in multiple injections over a 24 hr period.

† For comparative purposes, radioactivity excreted by control animals was set at 1000 for 3 hr collection period, and values for experimentals adjusted to these standards.

fully explained. While the changes in calcium excretion following parathyroidectomy were not as marked as the changes in phosphate excretion, the increased excretion was highly significant. In contrast, following parathyroid extract administration there was a small but significant drop in the rate of calcium excretion. To determine whether these changes occurred during the first hour as had been previously determined for changes in phosphate excretion(9), urine was collected separately for the second hour in several experiments. The total calcium values for urine collected this hour (Table II) indicate that the effect of parathyroidectomy on renal excretion of calcium was produced by the end of the second hour.

Renal calcium and phosphate excretion, hours 25-27. Table III summarizes calcium and phosphate excretion rates between 24th and 27th hours after start of experimental period. These data show that not only have the excretory rates for both ions returned to normal but that there appears to be a tendency to go to the opposite extreme. The only exception to this reversal was the continued high rate of excretion of phosphate following parathyroid extract administration.

Discussion. One of the most interesting conclusions from the data is that in addition to the long-recognized influence of the parathyroids on renal excretion of phosphate, these glands, at least in the rat, are apparently directly concerned with renal excretion of calcium. Renal calcium excretion rises more than threefold during the first hour after parathyroidectomy to an excretory rate that is maintained for the next 15 to 20 hours. During this period the serum calcium level falls steadily. Because of this increased and relatively constant excretory rate in the presence of a falling serum level, it seems logical to postulate that the increased renal excretion is at least partially responsible for the rate of drop in serum calcium level. Opposite effects on both serum and urine calcium occur following administration of parathyroid extract. These observations are strong evidence for concluding that calcium excretion changes produced are the result of a direct renal effect. In addition, the fact that these excretion changes are transitory indicates that the kidney threshold for calcium excretion has been changed; and that when the serum level becomes adjusted to the new threshold, calcium excretion returns to the normal rate. Subsequent changes are probably controlled by extra-renal influences on calcium entry or withdrawal from the blood. These experiments may aid in explaining the observations of Jahan and Pitts(14) who noted an increased tubular reabsorption as well as increased renal excretion of calcium following parathyroid extract administration to dogs.

Another very important result of this work is the further clarification of the role played by the kidney in the parathyroid control of phosphate metabolism. It would appear to be more advantageous to speak of the parathyroid control of the serum level of inorganic phosphate by means of changes in kidney threshold than to speak of the production of a constant phosphate excretion rate. In spite of the marked (up to 50 fold) drop in phosphate excretion immediately after parathyroidectomy, the excretion rate shortly (usually within 24 hours) returns to normal and in some cases rises above normal.

It is suggested, therefore, that the parathyroids exert both an extra-renal and a renal influence on calcium and phosphate metabolism. By the influence of the hormone of this gland on the kidney threshold the serum levels of both calcium and phosphate are maintained within their normal limits. Changes in titer of this hormone in the body cause transitory changes in calcium and phosphate excretion during the equilibration of serum levels with the new threshold limits. The major and more lasting effects of the parathyroids on excretion of either phosphate or calcium probably result from changes in the amounts of these ions available for excretion due to the extra-renal influences of this hormone.

Summary. This study has dealt with progressive changes which occur in renal phosphate and calcium excretion rates in rats during the first 27 hours after parathyroidectomy or the first injection of parathyroid extract. Following parathyroidectomy there is an immediate drop in urinary phosphate and a rise in urinary calcium. Within 24 hours the excretory rates for both these ions return to normal despite the continued high phosphate and low calcium levels of the serum. Following the initiation of parathyroid extract administration to normal rats the reverse is true. There is an immediate drop in urinary calcium and a rise in urinary phosphate. Twenty-four hours later the excretory rate for calcium returns to or rises above normal. The urinary phosphate, however, continues high throughout the entire period. These data are explained on the basis that immediate changes in renal excretory rates resulting from changes in parathyroid hormone titer are due to shifts in the renal threshold for these two ions. Excretion changes seen after the serum level has become adjusted to the new thresholds are considered to be due to extra-renal changes increasing or decreasing the entry of these ions into the serum.

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Effect of Intrinsic Factor Concentrate upon Utilization of Orally Administered Vitamin B_{12} by Rats.* (21354)

CHARLES ROSENBLUM, DAVID T. WOODBURY, ELIZABETH W. GILFILLAN, AND GLADYS A. EMERSON.

From the Research Laboratories, Merck and Co., Inc. and the Merck Institute for Therapeutic Research, Rahway, N. J.

The intrinsic factor activity(1-3) of gastric juice, hog mucosa concentrates and similar preparations in increasing the absorption of vit. B_{12} in pernicious anemia is currently evaluated by several non-hematological methods. Binding tests based upon microbial growth inhibition(4,5) or absorption(6-9), and upon dialysis(4,5) have been proposed, and have served a useful purpose in controlling isolation procedures(7,8,10). Such procedures lack specificity, however. It has furthermore been reported(11) that the binding power of various fractions of salt fractionated hog stomach concentrate is not proportional to their clinical activity. Measurements of fecal excretion(12) of cobalt 60 labeled vit. B_{12} administered orally to pernicious anemia patients in remission or to gastrectomized individuals(13) constitutes a more realistic approach to this assay problem. Other methods proposed for measuring intrinsic factor activity depend upon the hepatic uptake(14) or urinary excretion(15) (after injection of normal B_{12}) of the labeled tracer. Although the rat is not subject to pernicious anemia, contrasting distribution(16) and excretion(16-18) patterns are exhibited by these animals after oral administration and injection of 4-20 μg vit. B_{12}. Thus oral intake results in preponderant fecal elimination, with but slight retention in organs and excretion in the urine, whereas parenteral administration produces extensive incorporation in tissue and marked excretion in the urine, with but slight amounts present in the feces. Should intrinsic factor facilitate the absorption of vit. B_{12} from the stomach of the rat, as in the case of humans, such action would be reflected, at least qualitatively, in increased tissue and urinary B_{12} content. With this expectation in mind, vit. B_{12} was fed to normal and vit. B_{12}-deficient rats simultaneously with quantities of an intrinsic factor preparation twice that sufficient to bind the vitamin. Cobalt 60 labeled vit. B_{12} was employed to simplify the assay procedure. After 4 days, each animal was injected† with 20 μg of normal vit. B_{12} in an attempt to "flush out" (19) radioactive vitamin retained in the tissue. At the conclusion of the experiment livers and kidneys were removed for (radioactive) B_{12} assays. These organs retained(16,20) the highest percentages of administered vit. B_{12} and appeared to be target organs for the vitamin.

Contrary to our expectation, vit. B_{12} absorption by the rat was not increased by the simultaneous feeding of intrinsic factor. In point of fact, the added factor appeared to hinder absorption.

Experimental. Male rats of the Merck Institute strain, kept in metabolism cages, were placed at weaning on 2 diets, one a ration deficient in vit. B_{12} and the other supplemented with a high level of vit. B_{12} (400 μg/kg of ration). They were fed these diets for a period of 35 days prior to initiation of the experiments. The B_{12} deficient rats weighed an average of 180 g at the time of oral radioactive vitamin administration, whereas those receiving B_{12} averaged $\simeq$ 200 g at this point. The *composition* of ration employed in g/100 was: Soybean meal, 60; Wisconsin Salts (No. 4), 4; Dextrose, 24; Crisco, 10; Cod liver oil, 2. Micronutrients present in mg/100 g were:

* Presented at April 1954 meeting of Fed. of Am. Soc. for Exp. Biol.

† These experiments were performed many months before publication of the paper(15) by Dr. R. F. Schilling of the University of Wisconsin, in which humans are injected with crystalline vit. B_{12} 2 hours after oral administration of a 2 μg test dose. Perhaps earlier injection of our rats would have yielded greater urinary output, although it is doubtful that the pattern of results would have been altered significantly.

Thiamine, 1; Riboflavin, 2; Pyridoxine, 1; Calcium pantothenate, 10; Niacinamide, 10; Inositol, 5; Choline, 100; PABA, 30; Biotin, .05; Folic Acid, .2; Vit. E and K, 14.2 each. *Cobalt* 60 labeled vit. B_{12} with a specific activity of approximately 220 μc/mg was employed in these experiments. This preparation was established by radioactivity counter-current analysis(21) to consist of 94.3% B_{12} and 3.5% B_{12a}. The intrinsic factor sample employed was of hog pylorus origin, and was supplied by Aktieselskabet GEA, Copenhagen. The stated binding power of 500 mμg B_{12}/mg was checked by a modified dialysis method and by absorption on *L. leichmannii*(9) using radioactive vit. B_{12} as indicator. Clinical activity was also verified.‡ Aqueous solutions containing both components were prepared for dosing. One solution contained 4.2 μg of radioactive vit. B_{12} and 16.0 mg of intrinsic factor preparation/ml of which 1 ml was administered. A second solution contained 0.84 μg and 3.2 mg/ml of radioactive vitamin and intrinsic factor respectively/ml of which 0.5 ml was used for dosing. Two solutions containing only radioactive vitamin were also prepared to contain 8.4 μg/ml and 0.84 μg/ml respectively, and 0.5 ml volumes administered. The injection solution of pure crystalline (non-radioactive) vit. B_{12} was prepared to contain 80 μg/ml. One-fourth ml of this solution was administered to each animal. After a 2-day basal period of feces and urine collections, each of the 4 radioactive solutions, in the amounts indicated above, was administered by stomach syringe to a group of 4 rats from the set which had been maintained on the B_{12} deficient diet. A second set of 16 rats which had received the B_{12} supplemented diet was similarly treated. Thus each rat received either 0.42 or 4.2 μg of radioactive vitamin, with or without concomitant intrinsic factor. Feces and urine collections were made daily for 4 days after which each rat was given an intraperitoneal injection of 20 μg of normal vitamin. Daily collections were continued for an additional 4-day period, at which time the animals were anesthetized and sacrificed, and livers and kidneys removed for examination. *Kidneys and livers* were wet oxidized(22) in a mixture of nitric and sulfuric acids to yield clear solutions, which were evaporated to dryness and their residues taken up in dilute hydrochloric acid. Residues from small aliquots evaporated in 1″ stainless steel planchets were measured in a thin (mica) window Geiger counter. Feces samples were homogenized in water, and the entire suspension (50 ml) measured by gamma ray scintillation counting. Aliquots of urine specimens from rats receiving 4.2 μg of tracer vitamin (groups 3, 4, 7 and 8) were measured in a thin window counter after evaporation in 1″ stainless steel planchets. Urine samples from rats receiving 0.42 μg of vitamin (groups 1, 2, 5 and 6) were measured in a windowless counter after evaporation of aliquots in 1¼″ aluminum planchets. Appropriate corrections were made for self-absorption and coincident counts when necessary. The radio-activity per μg of tracer employed registered by the several instruments was $\simeq$ 56,800 cpm for thin window counter, $\simeq$ 14,300 cpm in the scintillation equipment, and $\simeq$ 322,000 cpm by windowless counting.

Results. Observations are recorded in the following tables in terms of % of administered radioactivity. Groups 2, 4, 6 and 8 received intrinsic factor which was withheld from groups 1, 3, 5 and 7. Animals are numbered from 1 through 32 to facilitate following the behavior of an individual subject. Rat 13 (group 4) regurgitated during administration of the test dose, hence was eliminated from the experiment. Furthermore Rat 29 (group 8) gave obviously abnormal values which were accordingly excluded from group averages. This abnormality is attributed to damage to the gastrointestinal tract, either inherent or caused during administration.

Table I lists results of the liver and kidney measurements. Part A of the table is devoted to rats maintained on the B_{12} deficient diet, and Part B to the rats receiving supplementary B_{12}. Group average radioactivity retentions by liver and kidneys are computed with average deviations from the mean and shown in the rows labeled "Avg % $\pm$ adm"; and these are followed by the equivalent μg vitamin cal-

‡ Courtesy of Dr. E. A. Reisner, Jr., New York University, Post Graduate Medical School.

TABLE I. Retention of Radioactivity* in Organs.

	B_{12} administered	.42 μg		4.2 μg	
	Intrinsic factor†	—	+	—	+
		A—Vit. B_{12} deficient groups			
	Group	1	2	3	4
Liver retention	%	(1) 1.91 (2) 1.44 (3) 2.25 (4) 1.71	(5) .76 (6) 1.48 (7) .14 (8) .29	(9) .21 (10) 1.19 (11) .57 (12) 3.69	(14) .77 (15) .54 (16) .17
	Avg % ± adm.	1.83 ± .25	.67 ± .45	1.42 ± 1.14	.49 ± .22
	Avg μg‡	.0077	.0028	.060	.020
Kidney retention	%	(1) 4.18 (2) 3.58 (3) 2.47 (4) 2.11	(5) 1.60 (6) 1.42 (7) .45 (8) .49	(9) .43 (10) 2.39 (11) .83 (12) 2.79	(14) .91 (15) 1.19 (16) .35
	Avg % ± adm.	3.09 ± .79	.99 ± .52	1.61 ± .98	.82 ± .31
	Avg μg‡	.0130	.0042	.068	.034
		B—Vit. B_{12} supplemented groups			
	Group	5	6	7	8
Liver retention	%	(17) .62 (18) 2.68 (19) .80 (20) 1.00	(21) 1.34 (22) .38 (23) .42 (24) .48	(25) .40 (26) .91 (27) .56 (28) .39	(29) (4.05)§ (30) .34 (31) .09 (32) .14
	Avg % ± adm.	1.28 ± .71	.66 ± .35	.57 ± .18	.19 ± .10
	Avg μg‡	.0054	.0028	.024	.0080
Kidney retention	%	(17) 4.64 (18) 12.92 (19) 6.12 (20) 8.19	(21) 6.80 (22) 2.56 (23) 4.93 (24) 5.50	(25) 2.21 (26) 3.29 (27) 2.90 (28) 2.05	(29) (26.7)§ (30) 1.81 (31) .84 (32) .63
	Avg % ± adm.	7.97 ±2.57	4.95 ±1.20	2.61 ± .48	1.09 ± .48
	Avg μg‡	.034	.021	.110	.046

* Thin window counter measurements.

† + sign indicates intrinsic factor administered with vitamin; — sign signifies intrinsic factor withheld.

‡ Calculated on basis that all radioactivity is present as vit. B_{12}.

§ These values excluded in computing group avg.

culated on the assumption that radioactivity represents administered vit. B_{12}.

Results of feces measurements are tabulated in Table II. Total radioactivity in collections for the first 5 days only are reported, since further elimination of radioactivity was negligible.

Table III is a report of urine radioactivity. Only the first 2 days post administration and post flushing were measured since the extent of excretion, small to begin with, was obviously diminishing. Total activities for the respective 2-day periods are reported.

Discussion. Inspection of Table I reveals that less radioactivity (presumed to be vit. B_{12} or proportional thereto) by a factor of 2-3, is retained by the livers and kidneys of the experimental animals when the intrinsic factor concentrate is administered along with the tracer B_{12}. This is true at both dosage levels and irrespective of the presence or absence of supplemental B_{12} in the diet. Presumably this is also true for other tissues, and indicates a lower absorption of B_{12} in the presence of intrinsic factor.

Application§ of the t-test method yields p values of 0.01 for the inhibitory effect of the intrinsic factor concentrate on B_{12} retention by the livers and kidneys.

In both the "deficient" and "B_{12} supplemented" rats, radioactivity retentions by the kidneys exceeded that of the liver with the exception of group 3. For the "deficient" rats, the kidney to liver ratio was $\simeq$ 1.5 whereas, in the case of rats on the B_{12} supplemented

§ The authors are grateful to Mr. F. W. Cleveland of Merck & Co., Inc., for the statistical evaluation of reported data.

TABLE II. Excretion of Radioactivity* in Feces.

	B_{12} administered	.42 μg		4.2 μg	
	Intrinsic factor†	—	+	—	+
		A—Vit. B_{12} deficient groups			
	Group	1	2	3	4
Excretion	%	(1) 71.0	(5) 69.2	(9) 51.7	(14) 29.7
		(2) 70.5	(6) 35.6	(10) 82.5	(15) 67.7
		(3) 72.4	(7) 93.2	(11) 38.8	(16) 32.6
		(4) 77.9	(8) 95.0	(12) 76.2	
	Avg % ± adm.	73.0 ± 2.5	73.3 ±20.9	62.3 ± 17.1	43.3 ± 16.2
		B—Vit. B_{12} supplemented groups			
	Group	5	6	7	8
Excretion	%	(17) 65.9	(21) 64.6	(25) 53.2	(29) (10.5)‡
		(18) 39.0	(22) 76.7	(26) 43.4	(30) 60.9
		(19) 79.0	(23) 64.8	(27) 73.2	(31) 82.4
		(20) 73.3	(24) 71.2	(28) 97.2	(32) 52.3
	Avg % ± adm.	64.3 ±12.7	69.3 ± 4.6	66.8 ± 18.5	65.2 ± 11.5

* Measured by scintillation counting.

† + sign indicates intrinsic factor administered with vitamin; — sign signifies intrinsic factor withheld.

‡ This value was excluded in computing the group avg.

diet, the ratio ranged from 4.6 to 7.5. This difference results from a lowered liver retention and increased kidney retention by animals maintained on the supplemented diet, and probably reflects a greater B_{12} content in the tissue of groups receiving dietary B_{12}. The higher B_{12} content of the kidneys and the lower liver B_{12} of the supplemented rats is in accord with previous observations(20) that kidney storage is greater, and liver and pancreas storage lower in saturated animals. The fact that the animals were flooded by injection of normal vit. B_{12} can have had but little effect on the above pattern, since this "flushing" was not performed until 4 days after oral administration of the test dose of labeled B_{12}; and the extent of urinary excretion resulting from injection, though significant, was slight. Apparently 4 days is too long a time lapse, and permits too firm fixing of absorbed B_{12} in the animal body, for flushing to be effective. In any case, translocation by a flushing-out process seems to be relatively unimportant(20) for organs such as kidneys, spleen and liver, although translocation from tissue such as skin and muscle is not excluded. This distribution pattern is independent of the presence of intrinsic factor, and must represent the behavior of absorbed vitamin.

The fecal excretion results shown in Table II are too variable, with too much overlap between groups to permit clear cut conclusions. No effect of intrinsic factor concentrate, either positive or negative, can be discerned. Fecal excretion of unabsorbed vitamin may have been incomplete, as has been noted elsewhere (23).

Urinary excretion (Table III) is low, as one would expect after oral administration(16, 17,23). Despite these low values, of the order of tenths per cent at most, the general pattern exhibited by the kidney and liver results is confirmed by the urinary output of radioactivity. It is to be noted that injection of 20 μg of normal B_{12}, 4 days after oral administration of the labeled vitamin, at which time excretion of radioactivity has ceased, actually liberates additional radioactivity, presumably in the form of vit. B_{12}. Although larger in general than the quantities excreted in urine prior to injection, the total excretion is still of a low order. The mechanism of this displacement effect is unknown, but may involve some sort of exchange process(24). The inhibitory effect of intrinsic factor may also be adduced from the urine result. This behavior is most noticeable in the post-flushing urines from rats fed 0.42 μg of labeled vitamin.

One may conclude from this investigation that the rat is not a suitable experimental animal for testing the ability of intrinsic factor to increase absorption of vit. B_{12}. Measurements

TABLE III. Excretion of Radioactivity in Urine.

	B_{12} administered	.42 μg*		4.2 μg†	
	Intrinsic factor‡	—	+	—	+
		A—Vit. B_{12} deficient groups			
	Group	1	2	3	4
1st 2 days post administration	%	(1) .0	(5) .0	(9) .01	(14) .02
		(2) .0	(6) .0	(10) .23	(15) .0
		(3) .0	(7) .0	(11) .27	(16) .0
		(4) .0	(8) .0	(12) .68	
	Avg % ± adm.	.0	.0	.30 ± .19	.01 ± .01
1st 2 days post flushing	%	(1) .82	(5) .31	(9) .06	(14) .07
		(2) .57	(6) .46	(10) .26	(15) .11
		(3) .47	(7) .06	(11) .06	(16) .03
		(4) .45	(8) .00	(12) .28	
	Avg % ± adm.	.58 ± .12	.21 ± .18	.17 ± .10	.07 ± .03
		B—Vit. B_{12} supplemented groups			
	Group	5	6	7	8
1st 2 days post administration	%	(17) .71	(21) .0	(25) .29	(29) (.25)§
		(18) .04	(22) .0	(26) .08	(30) .25
		(19) .07	(23) .0	(27) .13	(31) .00
		(20) .0	(24) .0	(28) .03	(32) .01
	Avg % ± adm.	.21 ± .25	.0	.13 ± .08	.09 ± .08
1st 2 days post flushing	%	(17) .40	(21) .38	(25) .12	(29) (1.84)§
		(18) 1.06	(22) .20	(26) .19	(30) .15
		(19) .31	(23) .24	(27) .07	(31) .05
		(20) .69	(24) .20	(28) .12	(32) .03
	Avg % ± adm.	.62 ± .26	.25 ± .06	.12 ± .03	.08 ± .05

* These urine residues measured with a windowless Q-gas counter.

† Thin window counter measurements.

‡ + sign indicates intrinsic factor administered with vitamin; — sign signifies intrinsic factor withheld.

§ These values excluded in computing averages.

of kidney, liver and urine radioactivities reveal an inhibition of absorption analogous, if not comparable in extent, to inhibition of microorganism growth by intrinsic factor preparations.

Summary. 1. The effect of a clinically active intrinsic factor concentrate on the absorption of cobalt 60 labeled vit. B_{12} by rats after oral administration has been studied. 2. Measurements of the radioactivity of kidneys, livers and urine indicate lowered absorption in the presence of intrinsic factor concentrate. Fecal radioactivity is too variable to permit reliable conclusions. 3. Injection of a massive dose (20 μg) of normal B_{12} 4 days after oral administration of the labeled vitamin liberates additional urinary radioactivity, and again points to an inhibitory effect of intrinsic factor concentrate.

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Destruction of Thyroid Gland of Atlantic Salmon (*Salmo Salar L.*) by Means of Radio-Iodine.* (21355)

GILLES LA ROCHE† AND C. P. LEBLOND.

From the Department of Anatomy, McGill University, Montreal.

Inasmuch as the follicles of the thyroid gland of salmon are scattered over a relatively large area along the ventral aorta, they cannot be extirpated surgically. However, in fish as in mammals, the gland takes up radio-iodine (1-5). It was, therefore, possible that large doses of this radio-element would destroy the thyroid gland of fish, as in laboratory animals (6) and man(7). Radioautographic studies of the thyroid region in salmon had revealed that injected radio-iodine can be traced into every single follicle (unpublished). It was, therefore, felt that large doses of radio-iodine would destroy even isolated follicles and thus produce complete thyroidectomy.

Large doses of radio-iodine were administered to young salmon at the parr stage to ascertain whether they could be thyroidectomized in this manner. The local and general effects of the treatment were then studied.

Method. The experiments were carried out in 1951 and 1952 at the Laurentide Fish Hatchery in St. Faustin, Quebec. Salmon parr were kept in tanks in which water was renewed at a rate of 4 l per minute. The water temperature was 5 ± 2°C during the winter period, increasing slowly in the spring to reach 10 ± 2°C in July and August. In a preliminary experiment, 4 salmon parr were given a single dose of 100 microcuries of radio-iodine intramuscularly and were sacrificed 2 months later. Since their thyroids still contained several intact follicles, it was decided to administer several successive doses of the isotope. The main experiment extended from Oct. 1951 to Aug. 1952. Salmon parr were divided into 4 groups of 32 animals each as indicated in Table I. Animals given radio-iodine were injected successively with 100, 50, 40, and 30

TABLE I.

Radio-iodine treatment	Diet	Mean body wt (g) Initial (Oct. 51)	At 5 mo (Feb. 52)	Final (Aug. 52)
No	Horse liver	34	40	59
"	*Idem* and thyroid	34	40	59
Yes	Horse liver	30	35	53
"	*Idem* and thyroid	32	37	59

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† Present address: Department of Biochemistry, University of Washington, Seattle.

microcuries of radio-iodine at the rate of one dose a month; the volume of fluid used per injection was kept below 0.2 cc. The other animals received equal volumes of physiological saline. All animals were fed minced horse liver, which in some of the groups (Table I) was mixed with an equal volume of thyroid powder prepared by drying and grinding whole beef thyroids. The fish of each group were weighed en masse about once a month throughout the experimental period as well as before autopsy. In Feb. 1952, one month after the fourth monthly injection of radio-iodine, 4 normal controls and 4 radio-iodine treated animals of corresponding weights were sacrificed by immersion in urethane. Histological examination of serial sections of their thyroid regions revealed an absence of thyroid follicles, and, therefore, the radio-iodine injections were stopped. The remaining animals were allowed to survive for another 6 months. The interruption of the experiment was due to the fact that in the summer of 1952, many of the radio-iodine treated animals were dying. Survivors of each group were then sacrificed and subjected to histological study.

Results. The glands of the normal control animals sacrificed either 5 or 10 months after the beginning of the experiment were composed of thyroid follicles with apparently normal epithelium and colloid. These follicles were either scattered among other structures or arranged in small groups (Fig. 1) along the ventral aorta. Examination of corresponding regions in radio-iodine treated animals showed loose connective tissue with occasional scarred areas, but without recognizable remnants of thyroid follicles (Fig. 2). Thus, the radio-iodine treatment had effectively eliminated all thyroid follicles. Hereafter, the injected animals will be referred to as "thyroidectomized."

The rate of growth was slow in all groups during the winter period, but accelerated with the approach of spring (Table I). The differences in growth rate between the thyroidectomized and intact groups were insignificant. The administration of thyroid extract did not affect growth.

The external appearance of the thyroidectomized animals indicated a diminution of pigmentation as compared to intact controls (Fig. 3-6). In a previous article(8) it was shown that pigment cells of the derma in salmon parr were responsible for the diffuse gray color of the fish, while deeper, hypodermal pigment cells were responsible for the dark areas known as lateral and dorsal bars. In thyroidectomized parr, the superficial diffuse pigmentation was generally discrete (Fig. 4-6) and the lateral bars appeared darker (Fig. 4) than in control animals (Fig. 3). However, when the lateral bars were exposed by scratching off epidermis and derma, they were of equal intensity in both groups. The apparent intensification of the lateral bars in most thyroidectomized animals may be due merely to the lightening of the surface pigmentation which normally obscures the lateral bars to some extent. Finally, the round spots observed dorsally and laterally appeared approximately equal in intensity in both groups (Fig. 5 and 6). Whether or not the pallor of the thyroidectomized animals was repaired by thyroid treatment could not be decided. This was due to the fact that by itself thyroid treatment in the doses used here produced a considerable degree of pallor in salmon parr(8).

After about 30 weeks, a few of the animals from the 2 thyroidectomized groups showed cutaneous erosions scattered along their back and sides. Four to 6 weeks later, as the temperature of the water reached 10°C, 24 of the 32 thyroidectomized animals fed on horse liver (3rd group, Table I) died. In contrast, the thyroidectomized animals receiving thyroid in the diet (last group) survived, as did the intact animals of the two first groups (Table I). The experiment was then discontinued.

Discussion. This work demonstrated that it is possible to thyroidectomize salmon by treatment with large doses of radio-iodine. Several injections of this isotope were necessary to insure completeness of the thyroidectomy.

Neither thyroidectomy nor treatment with thyroid extract influenced the rate of gain in body weight in salmon parr (Table I), in contrast to the striking effects of these procedures in mammals(9). Thyroidectomy appeared to decrease the superficial pigmentation of the parr.

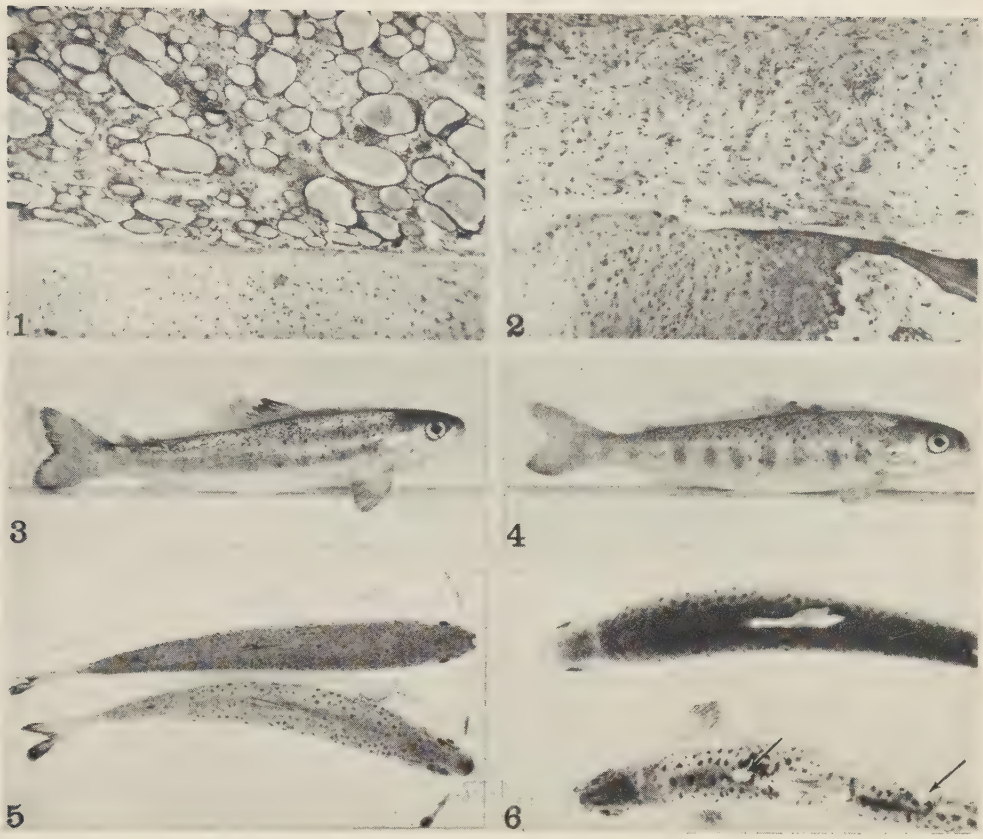

FIG. 1. Thyroid gland of a normal control salmon parr. 56 ×. The rather low epithelium and presence of abundant colloid within the follicles indicates a "normal" gland.

FIG. 2. Thyroid region of a salmon parr thyroidectomized by 4 injections of radio-iodine. 56 ×. The section was taken in same location as that of control animal in Fig. 1. Follicles destroyed by radio-iodine are replaced by loose connective tissue.

FIG. 3. Side view of a living normal control salmon parr. .31 ×.

FIG. 4. Side view of a living thyroidectomized salmon parr. .31 ×. Animal is more lightly colored than in Fig. 3, as seen in the tail region. In contrast, lateral bars are more prominent than in control animal in Fig. 3.

FIG. 5. Dorsal view of normal control (above) and thyroidectomized salmon parr (below) photographed under living conditions. .34 ×. Thyroidectomized animal is more lightly colored than control. However, round pigment spots on back are of the same intensity in both animals.

FIG. 6. Dorsal view of normal control (above) and thyroidectomized salmon parr (below) preserved in 10% formalin. .41 ×. Thyroidectomized animal is more lightly colored than control, although dorsal spots are of same intensity in both animals. In addition, small skin lesions may be distinguished in thyroidectomized animal (arrows).

The 4 groups survived in good condition through the winter whether or not they were given thyroid extract. However, when the temperature of the water had gradually risen to 10°C, most of the thyroidectomized animals which did not receive thyroid extract died. Cutaneous lesions were observed in these animals but were not believed to be a major cause of death, since the thyroid treated thyroidectomized animals showed the same lesions and survived. Rather the thyroidectomized animals seemed to have died as a result of their failure to adjust to the increased temperature of the water.

Conclusion. Salmon parr may be completely thyroidectomized by administration of repeated doses of radio-iodine(I^{131}). Preliminary observations indicate that the absence of the thyroid gland does not influence the rate of growth of these animals, but reduces the

superficial skin pigmentation and may impair the ability to survive a rise in water temperature.

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Technical Factors Influencing Permeability of Synovial Membrane in Rabbits.* (21356)

JOSEPH SEIFTER AND DAVID H. BAEDER.
From the Wyeth Institute of Applied Biochemistry, Philadelphia, Pa.

Our observation that hyaluronidase and steroids alter permeability of the synovial membrane of rabbits *in vivo*(1) has been confirmed by some investigators(2-5) and not by others(6-8). A critical examination of the methods employed by the latter invariably revealed a nullifying departure from the original technic. Since the method is not simple and permits little deviation we are presenting the results of a detailed study of the technical factors influencing it.

Method. In this study 2258 male rabbits of various breeds weighing 3.5 to 5.0 kg and free from obvious disease were housed singly in cages in soundproof quarters. They were fed Purina rabbit pellets and tap water *ad libitum*. Each rabbit received weekly an intramuscular injection of 300,000 units of procaine penicillin G to minimize respiratory and urinary bladder infection. Each rabbit was used only once every 7 days and not more than 5 times. Smaller rabbits were found to be unsatisfactory because they gave unpredictable excretion curves. The rabbits were anesthetized with 40 mg of Na pentobarbital/kg intraperitoneally and then secured on their backs to an animal board. The thong on the leg to be injected was placed distal to the talocrural articulation in order not to restrict circulation. A #12 Folley retention catheter lubricated with surgical jelly was inserted into the urinary bladder. The retention balloon was inflated with 5 ml water and the sidearm clamped for the duration of the test. Urine samples were obtained by flushing 5 ml physiological salt solution (PSS) through the irrigation tube. Excretion of dye was determined by the colorimetric method of Dandy and Rowntree(9). Injection into the synovial sac was through a 26 gauge 1″ needle inserted into

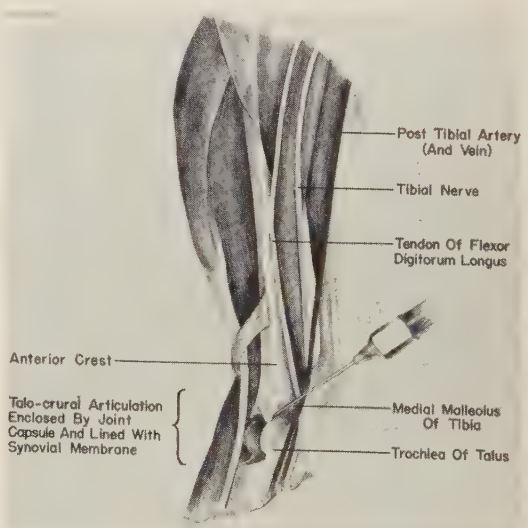

FIG. 1. Method of injection of talocrural articulation.

* Presented at meeting of Fed. Am. Soc. for Exp. Biol. and Med., Apr. 14-18, 1952.

the opening formed by the lateral and medial malleoli of the tibia-fibula and the tarsus of the talus (Fig. 1). When the point of the needle passed through the capsule and resistance ceased, injection was begun. A drop of synovial fluid could be withdrawn before injection when the needle was in proper position. The knee joint was injected with a 24 gauge needle inserted through the articular capsule. The injection consisted of 1.25 mg of Hynson, Westcott and Dunning clinical grade phenolsulfonpthalien (PSP) in 0.25 ml PSS. Unstandardized PSP or other dyes gave unsatisfactory results. Injection of various joints in approximately 300 rabbits indicated that *only the talocrural articulation could be used for a reliable test*. Interference with blood supply as would result from tying a thong proximal to the joint was obtained by inflating an infant size sphygmomanometer cuff wrapped securely about the thigh with one edge against the distal end of the femur. Each of the following pressures was applied to groups of 8 rabbits: 0, 10, 15, 17, 20 and 40 mm Hg. Local vasodilation was obtained in 6 rabbits by applying an ointment containing 0.25% methacholine chloride and 11% methyl salicylate over the joint area after the point of injection was covered with surgical adhesive tape. One hour later the tape was removed and the synovial sac injected. Local vasoconstriction was produced in 16 rabbits by exposure to air cooled by dry ice placed in a trough above the joint. Skin temperature over the joint as well as rectal temperature were recorded. PSP was injected 1 hour after exposure was started. The following schedule of administration before injection of PSP into the talocrural articulation was adhered to when the effect of various drugs on permeability was tested: steroids, intramuscularly, 60 minutes before; ACTH, intravenously, 30 minutes before; and hyaluronidase simultaneously into the joint.

Results. After mastering the method 2 technicians obtained the rate of excretion of PSP in 1958 rabbits of proper size receiving their first intra-articular injection. Erratic curves were obtained in 300 (15.3%). In 160 (8.2%) this was due to the following hitherto undescribed anomaly of the urinary bladder in which the urogenital sinus was found to be enlarged and the sphincter relaxed to permit entrance of the catheter into the seminal vesicle (Fig. 2). The remaining 140 failures could not be accounted for although incision of the joint proved the injection was accurately placed. These results indicated that with proper injection a failure rate of 15% could be expected. The failure to make a proper injection was less than 1% for both technicians.

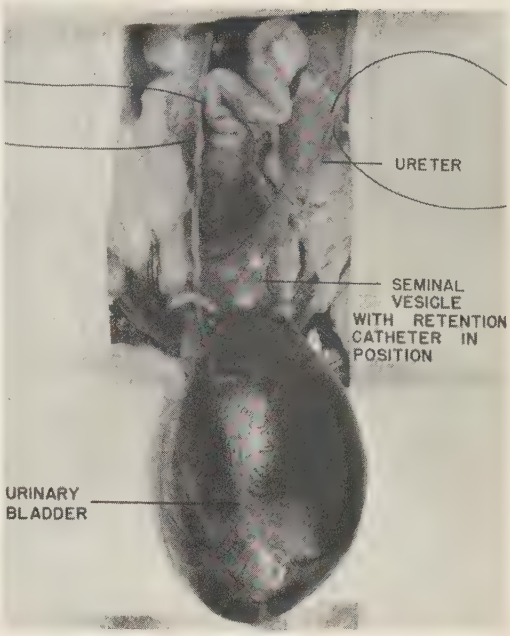

FIG. 2. Urinary bladder anomaly.

In the 1658 rabbits that had non-erratic excretion curves the mean rate of flow of PSP was 18 μg/minute (S.E. = $\pm$ 3.9%). Since

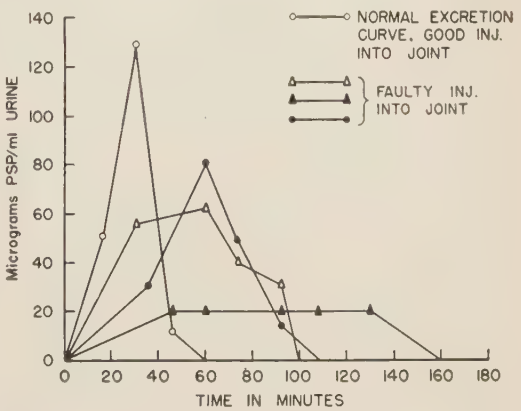

FIG. 3. Excretion patterns following faulty injection into talocrural articulation.

TABLE I. Effect of Interfering with Blood Supply to Joint on Excretion of PSP (Each Pressure = Avg 8 Rabbits).

mm Hg	Onset (min.)	Duration (min.)	Peak	μg PSP at peak	% PSP excreted
0	15	60	30	600	85 ± .9*
10	15	75	30	520	54 ± .7
15	15	135	30	120	53 ± .5
17	15	150	45	100	40 ± .4
20	15	150	45	95	33 ± .3
40	15	150	45	60	31 ± .2

* Stand. error.

the rabbits were standardized in 16 groups a random selection of the excretion curve of 1 from each group was made and the mean rate of flow was found to be 17.8 μg/minute (S.E. = ± 2.5%). A Student "t" test indicated no significant difference in response between groups.

After repeated injections into the same talocrural articulation, clearance of PSP was delayed significantly only after the fifth injection. At this time dye first appeared in the urine after 20 minutes instead of 15 as in the previous 5 injections and excretion was prolonged to 90 minutes instead of 60. The mean rate of excretion of PSP was reduced from 16 to 12 μg/minute. The standard errors were approximately ± 3.8% for all injections in the group of 10 rabbits. Faulty injection of the talocrural articulation resulted in erratic excretion as illustrated in Fig. 3 and resemble the curves published by Paul, Hodges, Knouse and Wright(7). Selection of the more easily injected knee joint in 10 rabbits resulted in variable onset of excretion (10-25 minutes) and equally variable duration (60-150 minutes.) The mean rate of excretion of PSP was 10.9 μg/minute with the S.E. ± 15.8%. The effects of interfering with the blood supply to the joint are shown in Table I. Progressive restriction resulted in progressive decrease in permeability of the synovial membrane. The appearance of dye in the urine was not delayed nor was the peak reached much later than in the controls; the excretion was significantly prolonged but the most striking effect was the decrease in concentration of dye at the peak excretion and in the total amount finally cleared. Table II shows that local vasoconstriction had similar effects. Vasodilation also prolonged excretion but did not decrease the amount of dye finally cleared across the synovial membrane. In the 16 rabbits there was no significant variation from an onset time of 50 minutes and a duration of 120 minutes. The mean excretion of PSP was 89% S.E. ± 0.2%. Massage of the site after injection of the synovial sac increased permeability sufficiently to counteract the effect of cortisone.

Discussion. Our original observation of the effect of a single dose of hyaluronidase, DCA, ACTH and cortisone(1) was confirmed by this method as is shown in Table III. These data and those obtained for 46 compounds selected at random from more than 300(10) which represent only a portion of the 5000 injections into the talocrural articulation of 1658 standardized rabbits were used to calculate the correlation value. The correlation obtained by 2 analysts is excellent ($r = 0.9$, $P < 0.001$).

Fig. 4 illustrates the precision provided the details are observed. A statistical analysis revealed that the effect of a compound on permeability could be tested on 2 rabbits at a P level of 95%.

A review of the methods used by the investigators who failed to confirm our results revealed the exact points of deviation. Edlund and Linderhold(6) used unselected rabbits, injected the knee joint, and had the needle in the joint constantly. Paul, Hodges, Knouse and Wright(7) also used unselected rabbits and injected the ankle joint with larger volumes in addition to administering the test compounds repeatedly. Hidalgo, McClure, Dean, Henderson, Whitehead and Smyth(8) used unselected rabbits, injected the knee joint, and tested the effect of chronic administration of

TABLE II. Effect of Local Vasoconstriction on Excretion of PSP (Avg 6 Rabbits).

Time (min.)	Temp., °C Skin*	Temp., °C Rectal	μg PSP/ml urine
0	10	39	0
25	0	39	15 ± .07†
45	—	—	20 ± .09
60	2	39	25 ± .12
75	—	—	18 ± .09
90	1	39	16 ± .08
105	—	—	8 ± .04
120	1	39	0

* Over joint area.
† Stand. error.

TABLE III. Collaborative Study on Synovial Permeability.

Treatment	Dose (mg/kg)	Route	Pairs of rabbits	Assayist A — Mean rate of flow*	Assayist A — S.E.	Assayist B — Mean rate of flow*	Assayist B — S.E.
ACTH	0.25	IV	7	0.7	.02	0.7	.02
Cortisone acetate	5	IM	5	1.3	.006	1.3	.006
DCA	5	IM	5	45	.2	45	.4
Hyaluronidase	150 TRU	T†	10	46	.3	46	.3

* μg PSP/min. † Into talocrural articulation.

compounds. In his review Gardner(11) concluded without experimental data of his own that these studies(6) were a valid basis for rejecting our findings. From the data of our studies it can be seen that the uncontrolled modifications by these workers invalidate their testing. The large number of experiments we performed establishes that the method is reliable and workable. The data are reproducible only when certain conditions are met, the most critical of which are accurate injection of the talocrural articulation and selection of rabbits for size and excretion pattern.

Delayed urinary excretion of PSP injected into the synovial sac of rabbits receiving ACTH or cortisone could be the result of several possible actions of these hormones. Alteration in renal function or systemic circulation was ruled out by determining the effect of ACTH and cortisone on the total clearance of dye 1 hour after intravenous injection of PSP. The mean excretion was 81.2 $\pm$ 1.3% in one group of 5 rabbits (A) and 82.5 $\pm$ 1.1% in another (B). One week later Group A received intramuscularly 5 mg cortisone acetate/rabbit and Group B intravenously 0.5 U ACTH/kg. PSP was then injected intravenously in all the rabbits (30 minutes for B and 60 for A). The mean excretion was not significantly altered; 82.8 $\pm$ 1.5% for A and 81.0 $\pm$ 1.7% for B. Evidence that cortisone and ACTH acted on the synovial membrane or its circulation was obtained by again treating A with cortisone and B with ACTH, injecting PSP into the talocrural articulation as usual and opening the joint 5 hours later to recover the unabsorbed dye. Group A had 68.4 $\pm$ 0.6% unabsorbed and 26.0 $\pm$ 0.3% excreted; Group B had 46.4 $\pm$ 0.8% unabsorbed and 50.2 $\pm$ 0.8% excreted. There appears to be no direct and unequivocal method at present for distinguishing *in vivo* whether the antipermeability effects of ACTH and cortisone were on the ground substance of the synovial membrane or on its blood vessels.

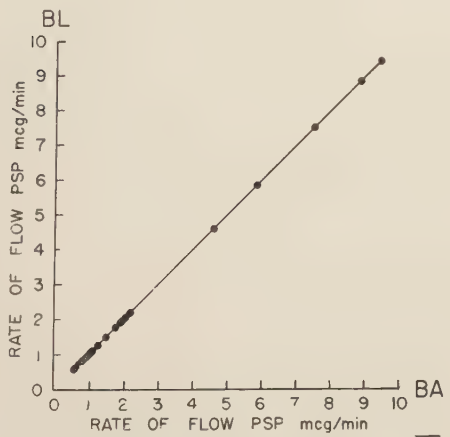

FIG. 4. Callaborative study between 2 assayists (46 compounds).

Summary. 1. A critical method for evaluation of synovial permeability based on more than 5000 intra-articular injections in 1658 selected rabbits is presented. 2. The method depends on accurate injection of the talocrural articulation, selection of animals for size and excretion pattern, and undisturbed circulation to the joint. 3. Two workers in the same laboratory using identical technics in testing the permeability effects of various compounds obtained data that show a high degree of correlation. 4. A hitherto undescribed urinary bladder anomaly in rabbits is noted.

We are indebted to Mr. Albert Blumenthal for assistance in this study.

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Effect of Adrenalectomy and Hypophysectomy in Permeability of the Synovial Membrane in Rabbits.* (21357)

DAVID H. BAEDER† AND JOSEPH SEIFTER.

From the Wyeth Institute of Applied Biochemistry, Philadelphia, Pa.

In previous studies it was shown that the rabbit synovial membrane offers a means for studying quantitatively the effects of steroids and other compounds on the permeability of connective tissue(1-3). The method used is accurate but does not differentiate between direct effects on the ground substance and stimulation of the pituitary-adrenal axis with release of anti-permeability substances. The purpose of the work reported in this paper was to determine in acute experiments the role of the pituitary and the adrenal on permeability of the synovial membrane and whether hypophysectomy or adrenalectomy would prevent the effects of certain steroids previously reported for intact rabbits(1,2).

Method. The method used was described by Seifter and Baeder(3). Adrenalectomy was performed in a 2-stage operation through the lumbar approach under sodium pentobarbital anesthesia. The adrenalectomized rabbits were maintained on the standard laboratory diet and their drinking water contained 0.9% sodium chloride. Successful adrenalectomy was confirmed in 2 ways: 1. All rabbits received ACTH (0.5 U/kg intravenously) and were used only if no anti-permeability effects were obtained. 2. At autopsy the lumbar region was examined for residual adrenal tissue. Hypophysectomy was performed by the method of Firor(4). The removed tissue was trapped in a gauze strainer and examined histologically. At termination of the experiment the completeness of operation was further established by examination of the sella turcica. The compounds studied were cortisone acetate, desoxycorticosterone acetate (DCA), ACTH, 21-acetoxy pregnenelone (compound 103), and allopregnane 21-ol-3, 20 dione 21 acetate (compound 125). The rabbits were used every 7 days for a maximum of 5 times. The effect of each compound was studied in 8 rabbits.

Results. The effect of adrenalectomy is shown in Table I. Seven days after adrenalectomy the maximum excretion of phenolsulfonpthalein (PSP) occurred in 45 minutes and persisted for 90 minutes as compared to 30 and 60 minutes pre-operatively. Statistical analysis revealed that these changes were not significant; however, there was a significant decrease in excretion rate during the first 30 minutes ($P = 0.01$). ACTH did not alter synovial membrane permeability in adrenalectomized rabbits which is in contrast to the anti-permeability effects in normal ones. The effects of cortisone acetate did not differ from that obtained in normal rabbits. After adrenalectomy compound 103 no longer suppressed synovial membrane permeability but

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† Portion of thesis submitted in partial fulfillment of requirements for degree of Doctor of Philosophy, Temple University, 1952.

TABLE I. Effect of Adrenalectomy on Synovial Membrane Permeability.

Treatment	Dose (mg/kg)	Route	Onset (min.)	Duration (min.)	Time of peak (min.)	Rate of excretion (μg PSP/min.)	± S.E., %
Standardization	—	—	15	60	30	18	.2
Adrenalectomy	—	—	15	90	45	11	.3
ACTH	0.5	IV	15	90	45	11	.3
Cortisone acetate	5	IM	30	270	*	1.3	.1
DCA	5	IM	10	30	10	41	.001
Hyaluronidase	150 TRU	T†	10	30	10	46	.06
Compound 103	5	IM	15	90	45	11	.3
" "	5	IM	15	90	45	11	.3
DCA	5	IM					
Compound 103	5	IM	15	90	45	11	.3
Hyaluronidase	150 TRU	T					
Compound 125	5	IM	25	110	60	7.4	.2
" "	5	IM	25	110	60	7.9	.3
DCA	5	IM					
Compound 125	5	IM	25	110	60	7.3	.3
Hyaluronidase	150 TRU	T					

* No peak. † Into talocrural articulation.

still antagonized the effects of DCA and hyaluronidase. The anti-permeability effect of compound 125 was not completely abolished by adrenalectomy, indicating a direct effect on the ground substance; the anti-DCA and "anti-hyaluronidase" actions were not significantly altered following adrenalectomy.

Effect of hypophysectomy is shown in Table II. The excretion rates obtained 7 days after hypophysectomy did not differ from those obtained pre-operatively. The effects of ACTH, cortisone, DCA, hyaluronidase, compounds 103 and 125 on synovial membrane permeability in hypophysectomized rabbits did not differ significantly from those obtained in normal rabbits.

Discussion. These studies indicate that cortisone, DCA, and hyaluronidase do not act indirectly through the adrenal-pituitary axis because these compounds had the same effect in adrenalectomized and hypophysectomized rabbits that they had in intact rabbits. This is in agreement with the findings of Winters and Flatiker(5) who studied the effects of these compounds on the spreading reaction in rats. It is assumed from these studies that compounds 103 and 125 appear to have 2 functions: 1. A stimulation of the adrenal cortex to release anti-permeability steroids. 2. An action on the ground substance directly to render it more resistant to the action of hyaluronidase and DCA. These results may account for the inconsistent clinical finding with the use of 21-acetoxy pregnenelone in rheumatoid arthritis(6,7). The effects of adrenalectomy to slightly depress permeability had previously been reported by D'Anunzo(8) who found in adrenalectomized rats the rate of excretion of methylene blue from the peritoneal cavity was decreased 40%.

It is obvious from these studies that compounds which have anti-permeability action in intact rabbits must be further tested in adrenalectomized ones to establish whether this action is the result of stimulation of the adrenal cortex or of a direct effect on the ground substance.

Summary. 1. Studies were carried out to determine the effects of adrenalectomy and hypophysectomy on permeability of the ground substance of the synovial membrane. 2. Neither adrenalectomy nor hypophysectomy had marked effects on permeability in short term experiments. 3. Of the compounds studied only cortisone acetate, DCA and hyaluronidase had unaltered effects on the permeability of the ground substance in the absence of the adrenals. 4. Both 21-acetoxy pregnenelone and allopregnane 21-ol-3, 21 dione 21 acetate were shown to have 2 actions. The anti-permeability action probably resulted from stimulation of the adrenal cortex. The

TABLE II. Effect of Hypophysectomy on Synovial Membrane Permeability.

Treatment	Dose (mg/kg)	Route	Onset (min.)	Duration (min.)	Time of peak (min.)	Rate of excretion (μg PSP/min.)	± S.E., %
Standardization	—	—	15	60	30	18	.2
Hypophysectomy	—	—	15	60	30	18	.2
ACTH	0.5	IV	45	30	*	.7	.3
Cortisone acetate	5	IM	30	270	*	1.3	.001
DCA	5	IM	10	30	10	41	.06
Hyaluronidase	150 TRU	T†	10	30	10	46	.06
Compound 103	5	IM	50	250	*	1.1	.001
" "	5	IM	50	250	*	1.0	.001
DCA	5	IM					
Compound 103	5	IM	50	250	*	1.1	.001
Hyaluronidase	150 TRU	T					
Compound 125	5	IM	30	210	*	.5	.001
" "	5	IM	30	210	*	.5	.001
DCA	5	IM					
Compound 125	5	IM	30	210	*	.5	.001
Hyaluronidase	150 TRU	T					

* No peak. † Into talocrural articulation.

antagonism to DCA and hyaluronidase was probably due to effects on the ground substance.

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Conjugates of Adrenal Corticoids in Human Plasma.* (21358)

ALFRED M. BONGIOVANNI, WALTER R. EBERLEIN,† MELVIN M. GRUMBACH,‡ JUDSON J. VANWYK,‡ AND GEORGE CLAYTON. (Introduced by J. L. Lilienthal, Jr.)

From the Department of Pediatrics, Johns Hopkins University School of Medicine and the Harriet Lane Home, Johns Hopkins Hospital, Baltimore, Md.

Much interest has centered on the estimation of the levels of circulating adrenal corticoids in the blood of man. The technic of Nelson and Samuels(1) for the measurement of "17-hydroxycorticosteroids" in blood has contributed greatly to the study of adrenocortical function. However, as originally described this method permits the determination only of the free (unconjugated) steroids. It was to be expected that conjugates of corticoids would be recovered from human plasma,

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† Children's Hospital of Philadelphia, University of Penna.

‡ Fellows of the National Foundation for Infantile Paralysis.

with appropriate technics, for two reasons. The majority of these compounds are present in human urine in conjugated form, presumably in great part as glucuronides. Conjugates of steroids have been extracted from the blood of dogs by Savard(2).

A method was devised for the extraction of conjugated corticoids from human blood(3). Preliminary findings have suggested the need for further study of such conjugates, since the results indicate that previous data(7) reporting only the free compounds, do not fully reflect the status of adrenocortical metabolism. Under some circumstances the results point to conclusions significantly different from those heretofore documented.

Methods. Samples of human plasma were directly extracted 3 times, each with 5 to 10 volumes of freshly distilled chloroform. This extract was evaporated, chromatographed on florosil and treated with phenylhydrazine according to Nelson and Samuels(1); this fraction was designated the "free" component. The remaining plasma was brought to approximately pH 5.5 with 40% sulfuric acid, and then to pH 4.5 with acetate buffer. Beta-glucuronidase (1000 units/ml of plasma) was added and the plasma was incubated at 37°C for 24 hours. Smaller quantities of glucuronidase were not effective and longer periods of incubation did not increase the yield under the conditions described. Following incubation, the hydrolyzed plasma was dialyzed at room temperature for 48 hours against 12 volumes of 10% methanol in water. The dialysate was extracted 3 times with ⅓ volume of chloroform. The chloroform extract was evaporated, chromatographed, and otherwise treated in the same manner as the free fraction. In addition to estimating the quantities of corticoids present with the phenylhydrazine reagent, large collections of plasma in some experiments were divided into several aliquots. These were studied with 2, 4-dinitrophenylhydrazine, and by separation on paper chromatograms according to Bush(4). Some aliquots were rechromatographed on silica gel, and the fluorescence attributable to the hydroxyl group at C 11 measured according to Sweat(5). Earlier trials with acid hydrolysis were unsatisfactory. The yields of material released were 25-40% of those measured on duplicate aliquots treated with glucuronidase. Furthermore, after hydrolysis the characteristics of the complexes formed with phenylhydrazine and dinitrophenylhydrazine measured with the Beckman DU spectrophotometer were not typical of those formed with known corticoids.

TABLE I. Control Values of Free and Conjugated Corticoids in Peripheral Blood of Subjects with Normal Adrenal Function, Adrenal Disease and Acute Stress.

Subject Sex	Age		Corticoids (γ/100 ml plasma) Free	Conjugates
♀	67	chronic pulmonary dis.	6.1	11.9
♀	13	rheumatic fever	9.2	9.2
♀	45	normal	6.0	7.7
♂	30	"	6.1	7.1
♂	32	"	6.0	3.2
♀	21	"	7.4	5.5
♀	26	"	5.5	3.9
♀	23	"	2.3	4.2
♀	11	adrenal carcinoma	34.2	10.5
♀	11	Cushing's syndrome	20.3	24.9
♀	20	" "	6.3	26.6
♀	13	" "	5.7	15.7
			19.7	15.2
			13.4	24.7
♂	25	acute head injury	25.0	29.0
♂	45	" peritonitis	21.0	18.0

Results. 1. Evaluation of the method. Due to the difficulties of obtaining corticoid conjugates similar to those in blood, and of duplicating the conditions under which such compounds occur normally in plasma, precise studies of recovery have been impracticable to date. Compounds measured in both the free and conjugated fractions behave in the manner of certain known corticoids when submitted to paper chromatography as detailed below. The incubation of glucuronidase alone with water, and treatment as above, did not yield material measurable by the technics employed. Substances such as penicillin or streptomycin were at no time added to plasma as preservatives, hence their possible contribution to materials released on hydrolysis was not a factor in these experiments. The only preservatives employed were toluene (1 ml or less) or simply the portion of chloroform remaining in the plasma to be hydrolyzed following the initial extraction for free compounds.

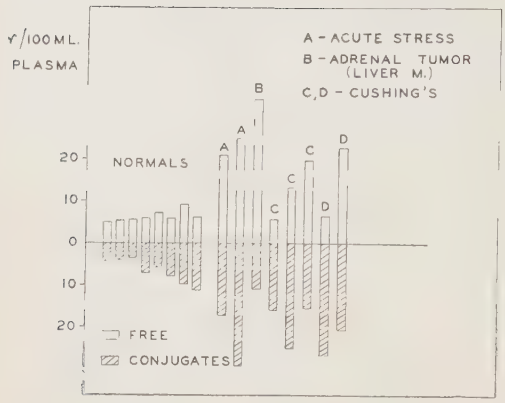

FIG. 1. Levels of free and conjugated Porter-Silber chromogens of normal subjects, patients under acute stress and adrenal disorders. All specimens were drawn between 9 AM and 10 AM.

2. *Levels in human plasma.* Table I and Fig. 1 indicate the levels of free and conjugated corticoids in the peripheral blood of 8 normal adults, two patients after acute injuries (A), of a child with an adrenocortical carcinoma with widespread metastases involving the liver (B), and five determinations on three patients with Cushing's syndrome (C and D). Three studies were conducted on a single patient with Cushing's syndrome due to adrenal hyperplasia (C) which illustrate the variability of the levels of free corticoids in this syndrome; however, the quantity of conjugated corticoids was uniformly elevated. The greatly elevated level of free corticoids in the patient with adrenocortical carcinoma (B) and the relatively small percentage of conjugated steroids may be related to the metastases to the liver demonstrated at autopsy. The normal range of free corticoids in plasma in this laboratory has been 2-10 gamma/100 ml (mean 8 ± 1).

3. *Administration of adrenocorticotropin and steroids.* In view of the elevations of both free and conjugated corticoids following stress, noted above, three studies were conducted following the administration of ACTH intravenously. The elevations in both free and conjugated moieties are demonstrated in 2 normal adults (Fig. 2). The rises were much more marked in a single case of Cushing's syndrome due to adrenocortical hyperplasia, wherein the conjugates were greater at 6 than at 4 hours; unfortunately the 6 hour free specimen was lost. *Compound E acetate* (17-hydroxy-11-dehydro-corticosterone) was administered by mouth to 10 subjects with normal adrenocortical and hepatic function. The quantities of free and conjugated corticoids found in the blood at intervals following various doses are detailed in Table II. At one hour the level of free steroids generally exceeded the conjugates, particularly at the higher dose levels. With 150 mg doses, the level of conjugated corticoids continued to show a steep rise up until 4 hours. With smaller doses the conjugated and free levels fell, but in general, beyond the 2 hour interval, the conjugated compounds exceeded the free, and frequently continued to rise although the free portion diminished. Compound E acetate was administered orally to 3 patients with cirrhosis of the liver. In Table II is shown the quantities of free and conjugated corticoids present in the circulating blood after the administration of 100 mg of compound E, in 5 normal adults and 3 patients with cirrhosis of the liver. In Fig. 3, detailed levels over a period of hours are indicated for 2 patients with cirrhosis and are compared to a single typical response in a normal subject. At no time is the percentage of conjugated steroids as great as in the normal, and the level of free compounds remains very high for a longer period of time in cirrhosis. The longer persistence of levels of free

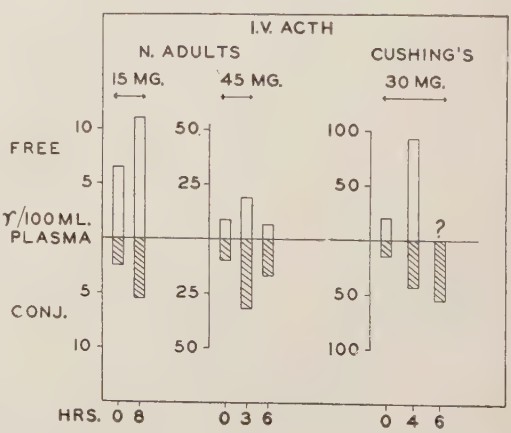

FIG. 2. Levels of free and conjugated Porter-Silber chromogens following administration of ACTH to 2 normal subjects and one with Cushing's syndrome, due to adrenal hyperplasia. Note differences in scale of ordinates.

TABLE II. Plasma Levels of Corticoids following Oral Administration of Compound E (17-hydroxy-11-dehydro-corticosterone) to Normal Subjects and Patients with Cirrhosis of the Liver.

	Hr	Plasma corticoids (γ/100 ml) Free	Conjugated
Normal adults			
1. C*		6.0	3.2
Comp. E 100 mg or*	1	25.8	23.9
	5½	5.8	10.3
2. C		7.4	5.5
Comp. E 100 mg or	1	58.7	23.9
	5½	5.8	10.3
3. C		3.9	3.2
Comp. E 100 mg or	1	103.0	—
	4	7.1	36.5
	6	2.6	27.7
4. C		8.7	9.7
Comp. E 100 mg or	1	64.0	71.6
	3	19.4	65.0
	5	6.5	38.8
5. C		6.0	7.7
150 mg comp. E or	1	69.0	26.0
	3	57.0	60.0
6. C		9.0	9.2
100 mg comp. E or	1	33.6	55.4
	6	9.0	9.2
7. C		11.3	1.6
150 mg comp. E or	1	94.2	39.8
	4½	12.9	97.3
	6½	8.1	69.6
8. C		6.0	3.2
75 mg comp. E or	1	25.8	23.9
	5½	5.8	10.3
9. C		7.4	5.5
100 mg comp. E or	1	58.7	78.1
	5½	6.0	19.4
10. C		8.7	9.7
100 mg comp. E or	1	64.0	71.6
	3	19.4	65.0
	5	6.5	38.8
Cirrhosis			
1. C		3.9	3.2
50 mg comp. E or	1	22.0	8.5
	4	21.0	23.2
2. C		8.1	1.9
100 mg comp. E or	1	43.2	8.1
	4½	50.3	18.4
3. C		3.2	0.0
100 mg comp. E or	1	36.8	7.4
	4	30.0	7.4
	6	23.2	2.0
	24	0.3	1.0

* C = Control; or = orally.

compounds suggests that preliminary conjugation of the corticoids by the liver is important

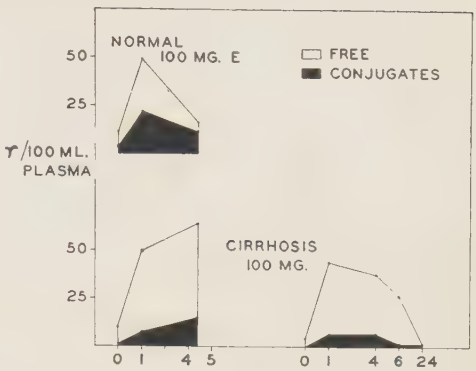

FIG. 3. Interval levels of plasma Porter-Silber chromogens in 2 patients with cirrhosis of liver compared with a single normal.

in the eventual disposition of these compounds. Further details are indicated in Table II. Subject No. 1 with cirrhosis had less liver impairment than 2 or 3, so that although there were less conjugated steroids at 1 hour, these rose significantly at 4 hours.

4. *Identification of compounds.* The conjugated material measured by the method described is believed to consist of corticoids for several reasons. On treatment with phenylhydrazine, there was almost always an absorption maximum at 410 mu characteristic of this group of compounds. Treatment with 2, 4-dinitrophenylhydrazine showed absorption maxima in the region of 470 mu. The conjugated fraction always demonstrated a marked rise following stress or the administration of ACTH and compounds E or F. In order to differentiate the compounds measured in the free and conjugated portions, fluorescence was measured using a mercury arc lamp with an exciting wavelength of 470-480 angstrom units and a transmission filter of 530-540, according to Sweat(5). The latter technique measures compounds B and F, having an 11 beta hydroxyl group and alpha-beta unsaturation in ring A. Sweat indicated that the reduced forms of F yielded only 4-14% of the fluorescence of compound F itself. Thus the fluorescence of compounds extracted were measured after the administration of compound F to 4 subjects. In the free fraction, the fluorescence corresponded to 60-100% of the quantity measured by phenylhydrazine, whereas in the conjugated portion it was 0-75%. This indi-

cates that the corticoids released by glucuronidase are in great part reduced as compared to the free fraction, although in the few studies conducted a portion of the free fraction was probably already reduced. In several instances, large quantities of blood were drawn from the subjects of these studies, and aliquots were chromatographed on paper according to Bush(4). The identity of the compounds was established by position (compared to known standards) and by reactions with blue tetrazolium, direct visualization under the ultraviolet lamp and following the methanolic-sodium hydroxide treatment. After stress or the administration of ACTH, the position and reactions of the free compounds on paper always conformed to those of compound F; tetrahydro F was found in a single extract of conjugated material following the administration of ACTH. Following the administration of compound F by mouth, it was found unchanged in the free fraction, although both tetrahydro-E and dihydro-F were present in small quantities on two separate occasions. In the single specimen studied, only tetrahydro-F was present in the conjugated fraction. In a single instance, following the administration of compound E by mouth, it appeared that only tetrahydro-E was present in the free fraction 4 hours later. To date it has not been possible to submit any of the material studied to infrared analysis since the quantities present are too small and very large volumes of human plasma would be required.

Discussion. It appears that conjugated corticoids are present in human plasma, and these may be extracted by proper technics. Preliminary investigations indicate that most of the unreduced (hence presumably active) corticoids reside in the free fraction, and that the conjugates contain predominantly reduced (dihydro- or tetrahydro-) forms. Although the latter may be physiologically inactive, they nonetheless faithfully mirror adrenocortical activity. Under certain circumstances, the free levels alone may not accurately indicate adrenocortical function. In the data presented, this might occur under circumstances of adrenocortical hyperactivity accompanied by the rapid conjugation of released steroids, as seems occasionally to be the case in Cushing's syndrome. Since tetrahydro-E was identified by paper chromatography in a single specimen of the free fraction, it appears that corticoids may be reduced prior to conjugation. The impaired conjugation of corticoids in human plasma noted in subjects with cirrhosis of the liver substantiates earlier studies(6) relating hepatic function to the conjugation of steroids. This relationship was also apparent in the single case of adrenocortical carcinoma with metastases to the liver, in which instance the proportion of conjugated corticoids to the greatly elevated total level, was low.

The study of the urinary corticoids alone may not reflect impaired conjugation in the presence of disease of the liver. Unless hepatic function is totally compromised, conjugation will proceed, albeit at a slower rate, and it is probable that the conjugated corticoids are excreted more readily by the kidneys. Hence one would not ordinarily find large quantities of free corticoids in urine.

Studies by others(7) have indicated a rapid fall in circulating free corticoids in man following the administration of compound E, so that peak levels were usually observed at one hour with diminutions in quantity thereafter. The data presented reveal a more sustained elevation with a gradual fall when the conjugates are taken into account. The more precipitous decline in free corticoids is no doubt due, in part, to the conjugation of the administered compounds. Further studies are contemplated of the free and conjugated corticoids in human plasma. Improved methods for the release and measurement of conjugated corticoids are being sought.

Summary. 1. It appears that conjugated corticoids (Porter-Silber chromogens) are present in human plasma which may be released by hydrolysis with beta-glucuronidase. 2. Under normal conditions the quantity of corticoids is approximately equal to the free. Under some circumstances, such as in Cushing's syndrome, the conjugates may exceed the free fraction. 3. Stress, ACTH or the administration of compounds E or F lead to elevations in the conjugated as well as the free fractions. The rate of disappearance of the free fraction is in part related to conjugation. 4. The rate of conjugation of corticoids in hu-

man plasma has been found to be defective in subjects with disease of the liver.

The authors are grateful to Doctor Lawson Wilkins for advice and suggestions. Some of the clinical material used was made available through the kindness of Doctors Harold Harrison and Roy Hertz. The beta-glucuronidase (Ketodase) employed was obtained from the Warner-Chilcott Laboratories, and is derived from beef liver.

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Occurrence of Glucose in Combination with Acetoacetate in Normal Urine. (21359)

M. C. NATH AND V. K. SAHU.*

From the University Department of Biochemistry, Nagpur, India.

It was postulated by Nath(1) that gradual accumulation of ketone bodies in the system might be responsible for the onset of hyperglycemia and the rise of blood sugar might be not only due to overproduction or nonutilization of glucose but also, as a result of a physiological response to combine, as a natural mechanism, with some toxic metabolites in the system. Subsequent work from this laboratory(2,3) has shown that repeated injections of acetoacetate in rabbits kept on Bengal gram (*Cicer arietinum*) diet could not only bring about hyperglycemia, through an initial stage of hypoglycemia, but also reduce glucose tolerance considerably. We(4) reported that although glucose and or acetoacetate can bring about a considerable rise in blood sugar when injected in normal rabbits, administration of the two, immediately following one another, is without such effect. That glucose reacts specifically with acetoacetate alone and not with B-hydroxybutyric acid, acetone or acetate(5), suggests the existence of a definite metabolic relationship between glucose and acetoacetate in the living body. This hypothesis has further been substantiated by our recent findings(5,6) that although glucose or acetoacetate individually fails to protect alloxan diabetes, administration of glucose immediately followed by its equimolecular amount of acetoacetate, or their condensation product, prepared and purified according to the method of West(7) prevents alloxan diabetes completely when administered even in very low concentration, prior to the injection of the diabetogenic dose of alloxan. This condensation product has been shown by Gonzales (8) and Gonzales and Aparacio(9) to be 2-tetrahydroxy butyl, 5-methyl, 4-carbethoxy furan.

Having obtained indirect proof of a metabolic relationship between glucose and acetoacetate, we looked for direct evidence of an *in vivo* combination of these 2 compounds in the normal animal. Before identifying a substance in animal tissues or in urine it was essential to find a reagent which might give a color reaction with the pure substance. $FeCl_3$ was suitable for determining even the minute trace of the condensation product (Na salt). It gives an orange color, varying in intensity with concentration of product in the solution. When a few drops of 10% $FeCl_3$ solution were added to normal urine (human) similar orange red color developed. This observation as well

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TABLE I. Free and Combined Glucose and Acetoacetate in Human, Rabbit and Rat Urines.

	Reducing sugar, mg/100 cc of urine			Acetoacetate, mg/100 cc urine estimated as acetone		
	Before hydrolysis	After hydrolysis	Amount liberated	Before hydrolysis	After hydrolysis	Amount liberated
Normal human‡	28.5 ± 1.32	51 ± 3.46	22.5	.173 ± .003	.344 ± .004	.171
Normal rabbits‡	32 ± 2.72	50 ± 2.25	18.1	.182 ± .004	.312 ± .004	.130
Normal rats‡	33 ± 2.3	53 ± 2.72	20.0	.190 ± .008	.315 ± .009	.125
Diabetic human			g			
Case I	8.5	8.5	0	10	10	0
Case II	7.5	7.5	0	5.42	5.42	0
Case III	6.8	6.8	0	4.90	4.92	0

‡ Each value is avg of 6 samples for normal urine.

as previous findings(10) that the condensation product of glucose and acetoacetate undergoes acid (HCl) hydrolysis of yield the original substances, prompted us to see if similar results could be obtained by carrying on the acid (HCl) hydrolysis of normal urine both from men as well as animals.

Experimental. 24 hour samples of urine were obtained from normal men, rabbits and rats, and also from diabetic persons (toluene being used as a preservative). A portion (15 cc) from each of filtered urine was then hydrolysed with 2.5 cc of conc. HCl by heating for about 7 minutes over a burner flame, neutralized with NaOH, filtered and made to 15 cc again with distilled water. All these hydrolysed urine samples reduced Nylander's reagent and yielded crystals of glucosazone though the same could not be obtained with any of the unhydrolysed urines. This indicated that free glucose is liberated from its combined form as a result of acid hydrolysis. Anthrone which affords a very sensitive test for glucose(11) and which shows a very faint positive test for the same in unhydrolysed urine, gives rise to a brilliant color to urine samples after hydrolysis. Further, every sample of normal urine when subjected to acid hydrolysis gives positive test for acetone, possibly formed from acetoacetate, which might be set free from its combined form with glucose, during the process of hydrolysis. Glucose was estimated according to the methods of Benedict and acetone by the Behre and Benedict method(12).

Results. Table I shows that although the concentration of acetone in urine samples was not proportional to that of glucose also liberated during the process of hydrolysis, the results are more or less comparable to those reported earlier(10) in the case of acid hydrolysis of the condensation product of glucose and ethyl acetoacetate.

As shown in Table II a much greater increase in acetone could be obtained if the acid hydrolysis was conducted at higher normalities of acid. But such acid concentrations cannot be used for the determination of glucose liberated, as it gets decomposed at higher normalities of acid. HCl hydrolysis, therefore, brings about a partial liberation of acetone from the complex product under such treatment. That B-hydroxybutyric acid did not split off any acetone during the above acid hydrolysis was also seen by carrying on actual acetone estimations with known amounts of B-hydroxybutyrate (Na salt) hydrolysed at different normalities of acid.

Several samples of urine from diabetic patients having high glycosuria and acetonuria were analysed as above and none indicated any rise either of glucose or acetone on hydrolysis with HCl.

It is thus evident that both glucose and ace-

TABLE II. Acetone Values of Normal Human Urine as a Result of Acid (HCl) Hydrolysis of the Same, Conducted at Different Normalities.

	Total acetone (avg values) in mg/100 cc urine		
Normality (N) of HCl	Before hydrolysis	After hydrolysis	Amount liberated
1.42	.173	.345	.172
5.0	.183	.624	.441
7.0	.175	2.300	2.125

toacetate occur in urine of normal subjects in a combined form. These data not only confirm our previous observation that there is a metabolic relationship between glucose and acetoacetate but also reveal an entirely new route of glucose utilization as well as of ketolysis.

It is also interesting to observe that the condensation product is absent in the diabetic human urine, suggesting a disturbance in the normal mechanism of combination of glucose with acetoacetate in diabetes, which may be an enzymatic one, going on normally in normal individuals.

Summary. (1) Glucose has been shown to occur in the combined form with some acetone body in the normal urine. (2) Both glucose and acetone can be set free from samples of urine from men, rats and rabbits on hydrolysis with HCl. (3) A new route of glucose utilization as well as ketolysis has been suggested.

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Growth-Promoting and Inhibiting Activity of Cholesterol Derivatives for *Attagenus piceus* "Yellows" (21360)

HERBERT MCKENNIS, JR.*
From the Department of Pharmacology, Medical College of Virginia, Richmond.

In a previous study(1) of the steroid requirement of *Attagenus piceus* "yellows," the comparative effects of cholesterol, 7-dehydrocholesterol and other cholesterol derivatives were examined. Cholesteryl chloride was found to inhibit the growth of larvae. This inhibition was overcome by increasing the cholesterol content of the diet. The antagonism observed is seemingly the first example of an anti-cholesterol effect produced by a structural analogue not of natural origin. Recently Nolan has reported(2) similar anti-cholesterol effects for cholesteryl chloride in *Blatella germanica* and in rabbits. In the original experiments with *Attagenus piceus* "yellows" cholesteryl acetate, the only cholesteryl ester tested, was found to possess growth-promoting ability. It seems desirable to study additional cholesteryl esters and other compounds for growth-promoting and growth-inhibiting ability.

Procedure. Newly-hatched *Attagenus piceus* "yellows," weighing approximately 0.5 mg, each, were incubated with 1.5 grams of finely-powdered diet at 32°-33° and 60% relative humidity in accordance with the previously described procedure(1). The basal diet had the following composition: Lactalbumin 7 HAAX 70, dried debittered brewer's yeast 7, corn starch 21, minerals U.S.P. XII (#2) 2. This diet is similar to diets employed by Moore(3). Cholesterol or other supplement was added to the basal diet before incubation. Protein in the basal diet was tested for choles-

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TABLE I. Effect of Cholesterol Esters and Other Compounds on Growth of *Attagenus* Larvae.

Group No.	Supplement to diet, mg/1.5 g diet			No. of larvae	Survivors at 7 wk	Avg wt (mg) gain of larvae
20- 1	Cholesteryl propionate		.115	20	20	1.6
20- 2				18	18	1.5
20- 3	"	butyrate	.118	16	16	1.3
20- 4				20	20	1.5
20- 5	"	*p*-toluenesulfonate	.14	18	18	1.3
20- 6				20	16	1.1
20- 7	"	benzoate	.132	22	22	1.0
20- 8				20	20	.7
20- 9	"	chloride	3.0	18	18	.2
20-10						
20-11	"	chloride	3.0	19	19	.4
20-12	"	butyrate	.0118	23	23	.3
20-13	"	chloride	3.0	18	18	1.4
20-14	"	butyrate	.118	20	18	1.7
20-15	Stilbestrol, .1 mg			21	21	.5
20-16				18	18	.4
20-17	No supplement			25	25	.5
20-18				19	19	.4

terol before use by Liebermann-Burchard reaction of a chloroform extract: 3 g of protein (No. 40 mesh or finer) was allowed to stand for 3 minutes with 20 ml of chloroform. If this chloroform extract did not give a positive test with the acetic anhydride-sulfuric acid reagent the protein was considered satisfactory for the basal diet. Some samples of commercial vitamin-free casein and other proteins have been found to respond positively. All such samples have been rendered cholesterol-free (in terms of the test) by extraction with isopropanol in a Soxhelet apparatus. Results of the growth and inhibition studies are shown in Tables I and II. The figures in each Table show the results of groups of replicates. Except for minor temperature fluctuations the groups in Table I are comparable to those in Table II.

The results indicate that lactalbumin in the presence of the other nutrients will support growth of *Attagenus* larvae. Minor variations in temperature do not permit more than a rough comparison with previous results obtained using casein as the source of protein.

At the levels examined, neither cholesteryl ethyl ether(4) nor dicholesteryl ether were effective in promoting the growth of larvae. Dicholesteryl ether synthesized by the method of Beynon, Heilbron and Spring(5) was of interest, since Silberman-Martyncewa have reported(6) the occurrence of this steroid in the spinal cords of cattle. Much cholesterol of commerce is derived from the latter source. Working with the cockroach, *Blatella germanica* L., Noland(7) found that cholesteryl, methyl, ethyl, propyl, and i-propyl ethers as well as dicholesteryl ether are incapable of supporting the growth of nymphs. Larvae of *Attagenus* "yellows" in our series gave similar results in that growth was not supported by the ethyl ether and dicholesteryl ether.

Cholesteryl chloride exerts an anti-cholesterol effect in both *Attagenus* "yellows"(1) and *Blatella germanica*(7), inhibition of growth which is overcome by additional cholesterol. In experiments here on *Attagenus* larvae, inhibition can similarly be overcome, in part at least, by addition of 7-dehydrocholesterol. Cholesteryl iodide did not support growth in view of cholesterol. A similar effect has been found with *Blatella*(7).

With respect to utilization of esters of cholesterol, *Attagenus piceus* "yellows" are less fastidious than *Blatella germanica* nymphs. The latter are reported by Noland to be incapable of using cholesteryl p-toluenesulfonate. In contrast (Table I) the former grew significantly in the presence of this ester. The acetate, propionate, benzoate and butyrate give positive response in both organisms (Table I)(7). The butyric acid ester also re-

TABLE II. Effect of Cholesteryl Ethers and Other Compounds on Growth of *Attagenus* Larvae.

Group No.	Supplement to diet, mg/1.5 g diet		No. of larvae	Survivors at 7 wk	Avg wt (mg) gain of larvae
22- 1	Cholesteryl ethyl ether	.1	16	16	.6
22- 2			17	17	1.0
22- 3	*Idem*	1	20	20	.7
22- 4			22	22	.8
22- 5	Dicholesteryl ether	.33	20	20	.7
22- 6			20	20	.8
22- 7	Testosterone propionate	1	21	21	.8
22- 8			20	20	.8
22- 9	Cholesteryl iodide	5	20	20	.6
22-10			22	22	.6
22-11			17	17	.7
22-12			19	19	1.0
22-13	" chloride	5	19	19	.1
22-14			19	18	.1
22-15	7-Dehydrocholesterol	5	18	18	2.6
22-16			22	22	3.0
22-17	7-Dehydrocholesterol	5	18	18	2.6
22-18	Cholesteryl chloride	5	18	18	1.7
22-19	Acetic acid, .2 ml 2M		18	18	.8
22-20			20	20	.9
22-21			19	19	.8
22-22			19	19	.8
22-23	No supplement		18	18	.9
22-24			19	19	.7
22-25			19	19	.8
22-26			20	20	.9

verses the inhibition by cholesteryl chloride.

In addition to cholesterol several compounds which have been implicated in cholesterol metabolism of higher animals(8,9) were examined for growth-promoting ability. Testosterone propionate, stilbestrol, and acetate ion were found not to support growth.

Summary. Cholesteryl propionate, butyrate, p-toluenesulfonate and benzoate have been found to support the growth of *Attagenus piceus* larvae in lieu of cholesterol. Neither dicholesteryl ether nor cholesteryl ethyl ether supported the growth of larvae. The toxic anti-cholesterol effect of cholesteryl chloride can be overcome in part by an equal quantity of 7-dehydrocholesterol.

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Effect of Cortisone upon Vascular Responsiveness of Potassium-Deficient and Normal Rats.* (21361)

Ray H. Rosenman, S. Charles Freed, and Malcolm K. Smith.
From the Harold Brunn Institute, Mount Zion Hospital, San Francisco, Calif.

A fall in blood pressure is observed in normotensive and hypertensive rats(1) (and in hypertensive humans(2)) following depletion of body potassium. Cortisone administration rapidly restores their lowered blood pressures to normotensive or hypertensive levels, respectively, without correction of their potassium-deficient state(3).

The mechanism of the cortisone-induced pressor effect in the potassium-deficient rat has not been clarified(4). It therefore was considered worthwhile to determine whether the vascular response to pressor agents of the potassium-deficient rat is increased by cortisone administration, in an attempt to explain in part the mechanism of the blood pressure rise induced by this steroid in such rats.

Material and methods. A series of 60 5-weeks-old male rats (Long-Evans) was fed a potassium-deficient diet(1). After 7 weeks, 20 of the rats (Group I) were subcutaneously injected with 2 mg of cortisone acetate twice daily, the remaining 40 rats serving as untreated controls. The blood pressure of each rat was obtained with the microphonic manometer prior to cortisone treatment and 7 days later. At that time the vascular reactivity of each rat was tested by the technic developed by Page and Taylor for dogs(5) and adapted for rats by Masson, Page and Corcoran(6). Under light ether anesthesia the abdomen was quickly opened and a small metal cannula inserted into the distal aorta and connected to a mercury manometer. The animal was then heparinized and the blood pressure allowed to stabilize. The test substances were injected into the exposed inferior vena cava through a fine hypodermic needle. Between each test injection an adequate time was allowed for return and stabilization of the blood pressure at the basal level. The rise of mean arterial pressure was thus determined following successive intravenous injections of nor-epinephrine (1 μg), methoxamine hydrochloride (Vasoxyl) (40 μg), and renin (0.5 mg). Each test dose was contained in 0.2 ml of solution. Similar studies were performed for further control purposes on a series of normal rats fed stock ration, 18 animals receiving cortisone (Group III) and 25 others remaining untreated (Group IV). The *effect of cortisone* upon vascular responsiveness was tested further in uninephrectomized rats with a renal ligature applied to the remaining kidney, since this procedure appears to "sensitize" rats to certain pressor drugs(10). A series of 5-weeks-old male rats was subjected to unilateral nephrectomy and application of a figure-of-eight ligature about the remaining kidney. The rats were fed stock ration throughout the experimental interval. Blood pressures were obtained postoperatively at weekly intervals with the microphonic manometer(7). Four weeks later, those animals which did *not* develop hypertension were used for further study. Cortisone was administered as in the previous experiment to 8 of the "renal-ligature" rats (Group I), the remaining 10 animals serving as untreated controls (Group II). After seven days of cortisone treatment the vascular responsiveness of each rat was tested following injection of nor-epinephrine (1 μg) and angiotonin (5 mg containing 2 units), as described above. Similar studies were performed for further control purposes on 16 uninephrectomized rats, of which 11 were treated with cortisone (Group III) and the other 5 remained untreated (Group IV). In a final experiment the vascular reactivity was tested in a small number of hypertensive rats treated with cortisone following preliminary depletion of body potassium. A series of 4-weeks-old rats was uninephrectomized and a figure-of-eight ligature applied about the remaining kid-

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TABLE I. Effect of Cortisone upon Vascular Responsiveness of Potassium-Deficient and Control Rats.

Group	Type of rat	No. of rats	Avg wt (g)	Avg blood pressure: Before cortisone	Avg blood pressure: After cortisone	Blood pressure rise induced by: Nor-epinephrine (Mm Hg)	Blood pressure rise induced by: Methoxamine hydrochloride (Mm Hg)	Blood pressure rise induced by: Renin* (Mm Hg)
				Rats fed potassium-deficient ration:				
I	Cortisone-treated	20	147	80 (68–94)†	112 (100–126)	31 ± 1.6‡ (20–42)	38 ± 1.7 (22–50)	61 ± 4.1 (46–72)
II	Untreated	40	154	83 (54–102)	81 (64–86)	24 ± .9 (14–36)	33 ± 1.3 (18–54)	44 ± 4.2 (26–70)
				Rats fed stock laboratory ration:				
III	Cortisone-treated	18	169	103 (90–118)	114 (70–142)	42 ± 1.9 (30–58)	52 ± 1.8 (38–64)	70 ± 2.6 (40–76)
IV	Untreated	25	178	108 (98–116)	106 (92–118)	36 ± 2.0 (18–50)	44 ± 2.1 (24–60)	69 ± 3.9 (56–82)

* 8 rats in each group. † Range of values. ‡ Stand. error of mean.

ney. After 4 weeks, 14 rats with persistent hypertension (over 140 mm Hg) were fed the potassium-deficient diet. Eight weeks later, half of the rats were injected subcutaneously, twice daily, with 2 mg of cortisone acetate, the remaining rats remaining untreated. After 3 days of cortisone treatment, the vascular response to renin was tested as described above.

Results. The vascular responsiveness of the intact potassium-deficient and the control diet rats is presented in Table I. As previously observed(1), chronic dietary deprivation of potassium (Group I) was associated with a lowering of the blood pressure and a somewhat diminished vascular reactivity to pressor substances (Group II), when the results obtained in these rats were compared with the values obtained in rats fed the control ration (Group IV). Cortisone administration rapidly restored the lowered blood pressures of the potassium-deficient rats (Group I) to normotensive levels, as previously noted(3); it also appeared to augment slightly but consistently their average rise of mean arterial blood pressure following injection of nor-epinephrine and methoxamine (Vasoxyl), the most marked effect being observed in the increased response to renin. The injection of cortisone in intact rats fed stock ration (Group III) appeared similarly to augment slightly their vascular response to nor-epinephrine and methoxamine, but not to renin, when the data in this group are compared with those observed in the untreated controls (Group IV).

Table II presents the results of the studies of vascular responsiveness in normotensive, uninephrectomized rats, with and without a renal ligature about the remaining kidney. In both types of animals the administration of cortisone appeared to augment their blood pressure response to angiotonin, only slight differences occurring in the average response to nor-epinephrine.

The final experiment concerned the effect of cortisone in potassium-depleted rats with renal hypertension. The average blood pressure rise induced by the test dose of renin in the cortisone-treated group of 7 such rats was 74 mm Hg (Range: 54 to 84 mm Hg). This contrasted sharply with the considerably lower average response of 25 mm Hg (Range: 14 to 42 mm Hg) in 7 similar rats which did not receive cortisone treatment.

Discussion. In previous studies(1) we have found that dietary deprivation of potassium ion in rats induces a specific hypotensive response, both in initially normotensive and in hypertensive rats. This depressor response is associated with depletion of body potassium and with a somewhat diminished vascular responsiveness to pressor drugs(8). The administration of cortisone to such potassium-deficient rats rapidly restores their lowered blood pressures(3) to their initially normotensive or hypertensive levels, respectively, despite persistence of their potassium depletion.

The mechanism of this "restoration" effect of cortisone has not been clarified(4). However, cortisone has been shown to induce augmentation of vascular tone(9), as well as vasoconstriction(10) and increased vascular responsiveness to nor-epinephrine(11). In the

TABLE II. Effect of Cortisone upon Vascular Reactivity of Uninephrectomized Rats and of Normotensive Rats with Renal Ligature.

Group	Type of rat	No. of rats	Avg wt (g)	Avg blood pressure Prior to cortisone (Mm Hg)	Avg blood pressure After cortisone (Mm Hg)	Avg blood pressure rise induced by: Nor-epinephrine (Mm Hg)	Avg blood pressure rise induced by: Angiotonin (Mm Hg)
	Uninephrectomized rats with renal ligature on remaining kidney:						
I	Cortisone-treated	8	302	127	132	31 ± 3.3* (20–52)†	103 ± 11.6 (76–124)
II	Untreated	10	300	125	136	25 ± 1.7 (10–34)	71 ± 4.9 (54–82)
	Uninephrectomized rats with intact remaining kidney:						
III	Cortisone-treated	11	222	105	118	33 ± 2.6 (18–46)	63 ± 2.4 (50–76)
IV	Untreated	5	210	104	113	28 ± 1.0 (24–28)	48 ± 5.1 (26–58)

* Stand. error of mean. † Range of values.

present study it again has been found that cortisone restores the lowered blood pressures of intact, potassium-deficient rats to normotensive levels. This appeared to occur in association with a slightly increased vascular responsiveness to injected pressor drugs. On the other hand, only slight differences were observed in the response to nor-epinephrine and to methoxamine in the cortisone-treated and the untreated potassium-deficient rats, and similar slight differences were noted in the cortisone-treated rats fed stock ration when compared to their respective control groups. Moreover, when these differences in the responses are analyzed statistically it can be seen that the standard errors of the differences in each instance are of a borderline nature. This suggests that the observed differences in responsiveness in the cortisone-treated rats are not very significant. Cortisone administration appeared to augment to a greater extent the pressor response to renin and to angiotonin in the potassium-depleted animals and in the uninephrectomized rats fed stock diet. However, statistical study in this instance again suggests that the data are not highly significant. Furthermore, no differences were observed in the responses to renin in the cortisone-treated, *intact* rats fed stock ration. Finally, the technic used to assay vascular responsiveness is not a highly critical one in small animals such as rats, and a wide range of values are observed in the data.

It seems likely that the effect of cortisone upon the blood pressure of hypotensive, potassium-deficient rats is not significantly related to an ability of cortisone to increase vascular reactivity. This conclusion is in agreement with the studies of Williams *et al.*(12) in intact rabbits in which it was found by direct observation that cortisone did not augment the arteriolar sensitivity to nor-epinephrine. Griffith and his associates(13) also failed to find any augmentation of pressor responsiveness by cortisone in humans with shock associated with myocardial infarction. Indeed, Williams and associates(12) found in their studies of the ear vasculature of intact, normal rabbits that cortisone did augment vascular tone without increasing sensitivity to nor-epinephrine.

Summary. Hypotension was induced in intact rats by dietary deprivation of potassium. Cortisone administration to such rats rapidly restored their blood pressures to normotensive levels. In these animals as well as in intact and uninephrectomized rats with and without a ligature about the remaining kidney and which were fed stock ration, cortisone did not appear to alter *significantly* their vascular responsiveness to pressor drugs. The data in this study suggest the restorative effect of cortisone upon blood pressure of potassium-depleted, hypotensive rats is not accounted for by augmentation of vascular responsiveness to pressor substances.

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Effect of Selenium on Reproduction in Rats.* (21362)

IRENE ROSENFELD AND O. A. BEATH.†

From Agricultural Research Chemistry Department, University of Wyoming, Laramie.

The effect of selenium on reproduction in mammals has not been fully investigated. Many investigators noted decreased fertility and malformations during selenium intake in different species of animals. Munsell, DeVaney and Kennedy(1) studied the effect of wheat containing selenium in diets of rats. They reported that wheat selenium had detrimental effect on growth and reproduction in direct proportion to the selenium intake. Franke and Potter(2) used toxic selenized wheat in the diet of rats and studied the survival and reproduction in white rats. They observed that rats which survived the toxic diets for a long time showed subnormal growth and distinct loss in reproductive power. Our studies were initiated to obtain data on the effect of varying concentration of inorganic selenium in drinking water on reproduction in successive generations of rats, this report presents the results of these studies.

Method. Wistar strain white albino rats were used as experimental animals. All animals were fed with Purina laboratory chow during the experiment. Groups of 5 pregnant females were placed on selenium at the beginning of the experiment. Selenium was added as potassium selenate to drinking water in the following concentrations: 1.5; 2.5; and 7.5 ppm of Se. Five pregnant rats served as controls. All animals at the beginning of the experiments were approximately the same age and weight. The experiments were continued for one year. Each control and experimental animal was bred 5 times during the experiment. The selenium content of the water in each group was maintained at the same concentration during the entire experiment.

Results. Rats on 1.5 and 2.5 ppm of Se in water had normal litters. This concentration of selenium in water had no effect(1) on the reproduction of breeding rats, (2) the number of young reared by the mothers, or (3) on the reproduction of two successive generations of males and females. The second generation of selenium rats which received 2.5 ppm of Se in water had normal offspring but the number of young reared by the mothers decreased about

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TABLE I. The Effect of Selenium on Reproduction.

Group	No. rats ♂	No. rats ♀	Duration Se intake (mo) ♂	Duration Se intake (mo) ♀	Se intake (ppm) ♂	Se intake (ppm) ♀	Total No. offspring	Total No. survived	Remarks
I	2	5	0	0	0	0	250	230	Controls mated 5 times.
II	3	5	1	1	7.5	7.5	60	8	Selenium given 5-8 days before parturition.
III	3	5	8	1	7.5	7.5	21	4	Selenized ♂* mated with ♀ which received selenium during mating and after parturition.
IV	3	5	8	0	7.5	0	55	38	Selenized ♂* mated with normal ♀.
V	3	5	1	8	7.5	7.5	0		♂ received selenium during mating with selenized ♀.*
VI	3	5	0	8	0	7.5	0		Normal ♂ mated with selenized ♀.*

* Males and females received 7.5 ppm Se since birth.

50 per cent. The weight of the females in this generation was 20 ± 10 g less than that of normal rats of the same age.

As the concentration of selenium was increased to 7.5 ppm in water, there was decrease in fertility, decrease in the number of survivals, reduction in the rate of growth in the young, loss in weight in the adult animals, and finally death due to chronic selenium poisoning.

The results on the effect of selenium on reproduction under varying experimental conditions are given in Table I. Group II in Table I gives the effect of selenium on pregnant rats which received 7.5 ppm Se in water from 5 to 8 days before parturition. The selenium intake at this concentration had no effect on the young before birth. The rats had normal litters but there was a decrease in the number of survivals with the continued selenium intake and only 13 per cent of the suckling rats survived. McConnell(3) reported the passage of selenium into the milk by the use of radioactive selenium.

After the birth of the litters the selenium intake in the mothers was continued for 11 months. These selenized females were mated 5 times with selenized males but failed to reproduce. The females in this group lost considerable weight during the experiment. At the end of 12 months 3 females and one male died from chronic selenium poisoning.

The growth rate in rats which received 7.5 ppm of Se since birth was considerably slower than in normal rats. At the end of 6 months the young females weighed on the average about 125 g and the males 246 g. There was some increase in weight of the young rats but the average weight of females which survived the one year experimental period was 80 ± 20 g less than normal females. The average weight of males was 60 ± 10 g less than that of normal rats of the same age. The males and females in this group were not separated for 8 months, but failed to reproduce. In order to determine whether the failure of reproduction was due to the effect of selenium on the males or females, normal females were mated with selenized males and normal males were mated with selenized females.

The results on the effect of 7.5 ppm of Se in water on the reproduction using selenized males with females which received selenium during mating and parturition and females which received no selenium but were mated with selenized males are given in Table I, Groups III and IV. Two females from the 5 rats which were placed on selenium had litters within 7 to 10 weeks. The newborn rats appeared normal but only 4 of the offspring from the litters were reared by the mothers. Westfall *et al.*(4) demonstrated the placental transmission of selenium in early, midterm, and late pregnancy in rats. Our results indicate that survival of the progeny was influenced by the selenium intake of the mothers.

The young rats in this group also showed decrease in the rate of growth and reduced fertility similar to the earlier group. The above breeding males and females were continued on selenium but they failed to have a second litter and the females lost about 50 g of weight at the termination of the experiment. Normal females mated with selenized males had normal reproduction and survival, as indicated in Table I, Group IV.

When selenized females were mated with normal males whether the selenium intake was continued or whether it was omitted during breeding, the females failed to reproduce as indicated in Table I, Groups V and VI.

These results indicate that at this concentration of selenium the failure of reproduction in the rats was due to the effect of selenium on the females. Fertility in the males was not affected by 7.5 ppm of Se. Whether the inability of females to reproduce was related directly to the greater sensitivity of the females to the toxic effects of selenium and subsequent damage to the reproductive organs or whether it was due to the effect of selenium on the estrus cycle cannot be stated at the present time. Histological sudies on the male and female reproductive organs will be carried out in the near future in order to determine the extent of damage by selenium to these organs.

Summary. Low concentration (1.5 and 2.5 ppm) selenium in drinking water for two generations had no effect on the reproduction in rats. 2.5 ppm of Se reduced the number of young reared by the second generation of mothers. 7.5 ppm selenium prevented reproduction in females but fertility of the males was not affected.

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Comparative Study of Lipids in Vertebrate Testes.*† (21363)

R. M. MELAMPY AND L. F. CAVAZOS.‡

From the Iowa Agricultural Experiment Station, Iowa State College, Ames.

A variety of lipids are recognized as important constituents of protoplasm and it is possible through histochemical procedures to localize certain of these substances in tissues. According to Gomori(1) histochemistry of lipids is difficult and often illusory. However, the information obtained is useful in evaluating the role of lipids in cellular composition and function. This investigation deals with morphological distribution of various lipid components in the testes of sexually mature individuals representing the classes Mammalia, Aves, Reptilia, Amphibia, and Pisces of the subphylum Vertebrata. A similar comparative study of the periodic-acid reactive carbohydrates has been made by Cavazos and Melampy(2).

Materials and methods. In the class Mammalia, testes were fixed from 2 bulls, 4 rams, one boar, 9 rats, and 2 guinea pigs. Two New Hampshire Red roosters represented Aves. In Reptilia, 8 horned lizards, *Phrynosoma cornutus* (Harlan), were studied. Eighteen grass frogs, *Rana pipiens,* and 12 bluegill fish, *Lepomis macrochirus* Rafinesque, were used as examples of classes Amphibia and Pisces respectively. Tissues were fixed in 10% neutral formalin, washed in tap water for 6 hours, placed in gelatine at 37°C overnight, and embedded

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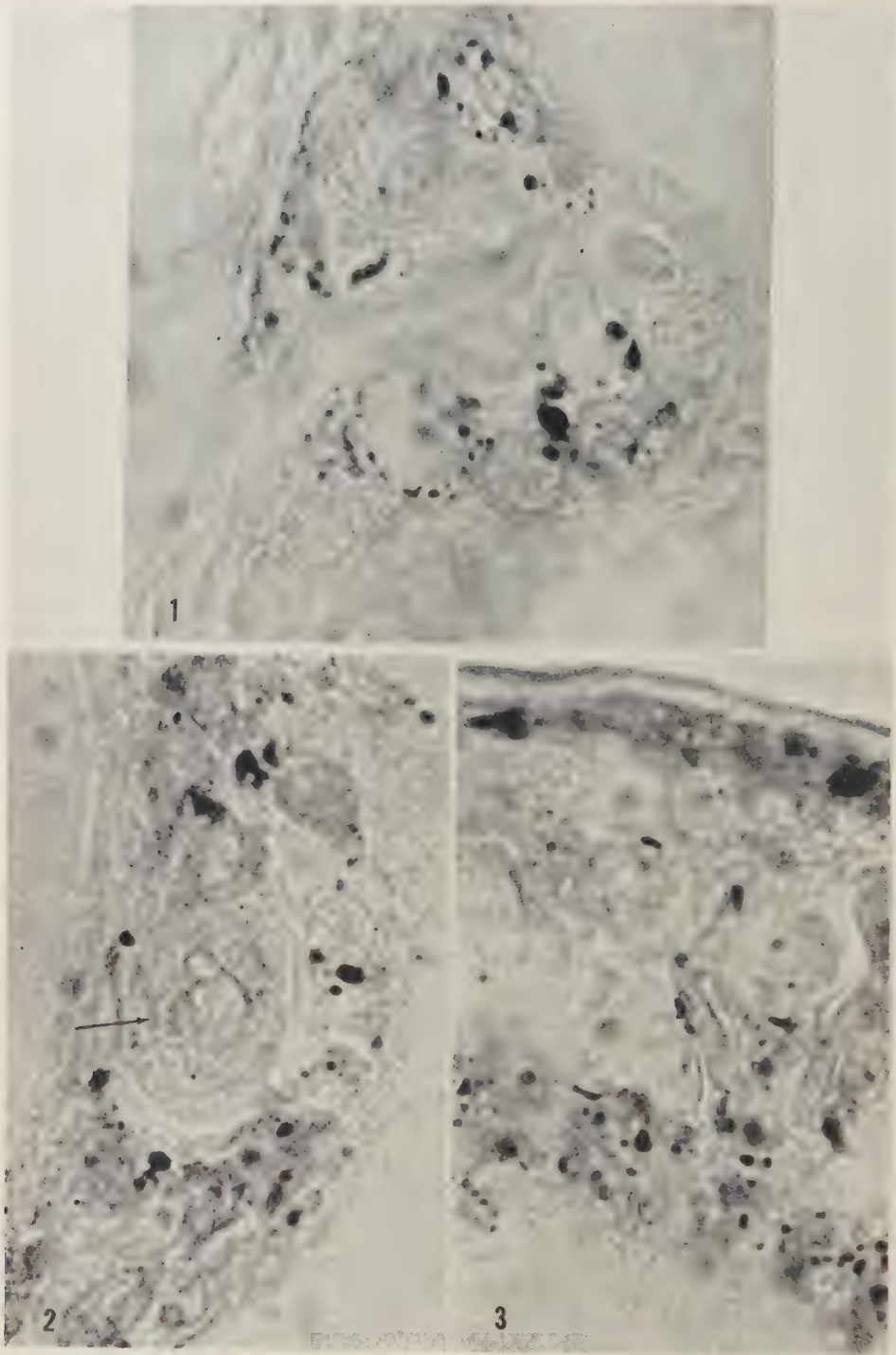
‡ Present address: Department of Anatomy, Medical College of Virginia, Richmond.



×90 objective and ×7.5 ocular

FIG. 1. Sudan black B-reactive droplets in interstitial cell cytoplasm of bovine testis. Note presence of unstained nuclei. FIG. 2. Lipid particles in cytoplasm of ram Leydig cells. Fixation in neutral formalin and staining with Sudan black B. Arrow indicates an arteriole containing unstained erythrocytes. FIG. 3. Intratubular lipids in the seminiferous tubule of the ram following Sudan black B. Large sudanophilic droplets are present along basement membrane of tubule.

in 10% gelatine. Sections were cut at 10 μ on a freezing microtome, and the following histochemical methods were applied: (a) Sudan black B, using procedure of Herman(3); (b) Oil red O counterstained with Mayer's hemalum as suggested by Lillie(4); (c) Seligman and Ashbel method for carbonyl groups (5); (d) Schultz reaction for cholesterol and its esters(1), and (e) polarizing microscope used for birefringence(1). Sections were treated with digitonin as this substance forms insoluble, anisotropic esters with cholesterol and other β-steroids(6). Control sections were prepared by treatment with a mixture of equal parts of ethyl ether and chloroform at room temperature for 30 minutes to 48 hours. Treatment of control sections with fat solvents did not always completely remove reactive materials, and this may be related to the protective action of proteins as suggested by Gomori (1). Sections to be used as controls for polarization microscopy were extracted in acetone overnight prior to treatment with digitonin. Testicular material, from all species studied except fish, was fixed in formaldehyde calcium and in weak Bouin's fluid as recommended by Baker(7) for phospholipid.

Observations. Treatment with Sudan black B and oil red O. Following application of these stains to bull, ram, rat, guinea pig, chicken, lizard, and frog testes, it was observed that nuclei of interstitial cells were unstained, whereas the cytoplasm showed rather heavy concentrations of lipid in the form of fine and large droplets (Fig. 1, 2, 4, 6, and 9). In some cases many of the larger globules were vacuolated. In the case of the boar, only a few fine lipid granules were demonstrated in the cytoplasm of these cells. In the basement membrane of the seminiferous tubules of the bull and boar, a slight reaction was noted. As shown in Fig. 3, a moderate sudanophilia was demonstrated in the ram, whereas in all other species studied, with the exception of the fish, this reaction was strong. In the bluegill, there were no structures comparable to the basement membrane or the Sertoli cells. In the last-mentioned species, a weak reaction was observed in the testicular sheath of connective tissue which invested the testis and in the interlobular connective tissue. In the cytoplasm of Sertoli cells, fine and large granules, some vacuolated, were found in the bull, ram (Fig. 3), rat, chicken, lizard, and frog. In the guinea pig this reaction was somewhat weaker, whereas in the boar little, if any, reacting lipid was present in these cells. The nuclei and nucleoli of the Sertoli cells remained unstained in all species examined. Within the seminiferous tubules, a strong sudanophilia was observed in the spermatogonia, as well as in the primary and secondary spermatocytes and spermatids of the bull, chicken, and lizard (Fig. 8). This reaction was weak in all the other species examined. Occasionally, in the bull, ram, and chicken spermatocytes an unstained cytoplasmic area was observed adjacent to the nucleus. Likewise, sudanophilic material was localized at times in the Golgi region of the spermatocytes and spermatids of the fowl, horned lizard, frog, and bluegill. In the rat, lipids were often observed in the Golgi zone of the spermatids. However, it must be emphasized that the amount of lipid was quite variable within the same cell types (Fig. 1, 2, 4, 6, and 9), between the different species, and even among individuals of the same species.

Carbonyl groups. Positive staining, generally as fine cytoplasmic droplets, was observed in the Leydig cells of all species examined, with the exception of the boar, chicken, and fish. As seen in Fig. 5, 7, and 10, the amount of reactive substance present in these cells was quite variable, and in many instances the interstitial cells were negative. The strongest reaction was noted in the case of the guinea pig (Fig. 7) and amphibian, in which fine as well as large granules were seen.

Phospholipid. A strongly positive coloration indicating presence of phospholipid was noted in cytoplasm of interstitial cells of the lizard and frog (Fig. 11). Moreover, excepting the ram, which demonstrated a weak response in the Leydig cells, a moderate amount of reactive material was observed in the other species. Fine droplets containing phospholipid were also observed in the interstitial cells. The nuclei were negative; however, in the frog the nucleoli of the Leydig, Sertoli, and germ cells were deeply stained (Fig. 11). The Sertoli cells of the bull, ram, rat, guinea pig, and fowl contained moderate quantities of fine re-

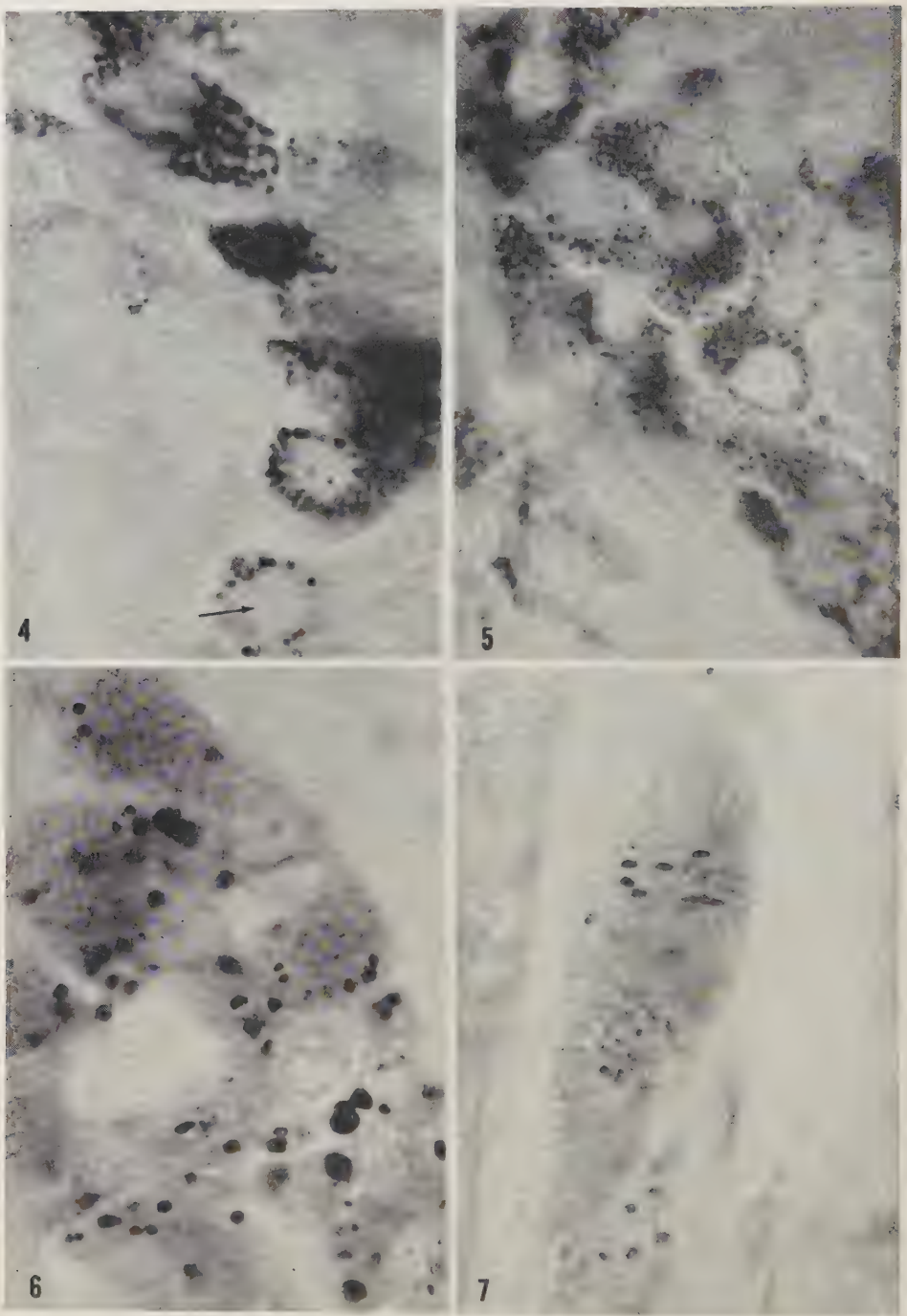
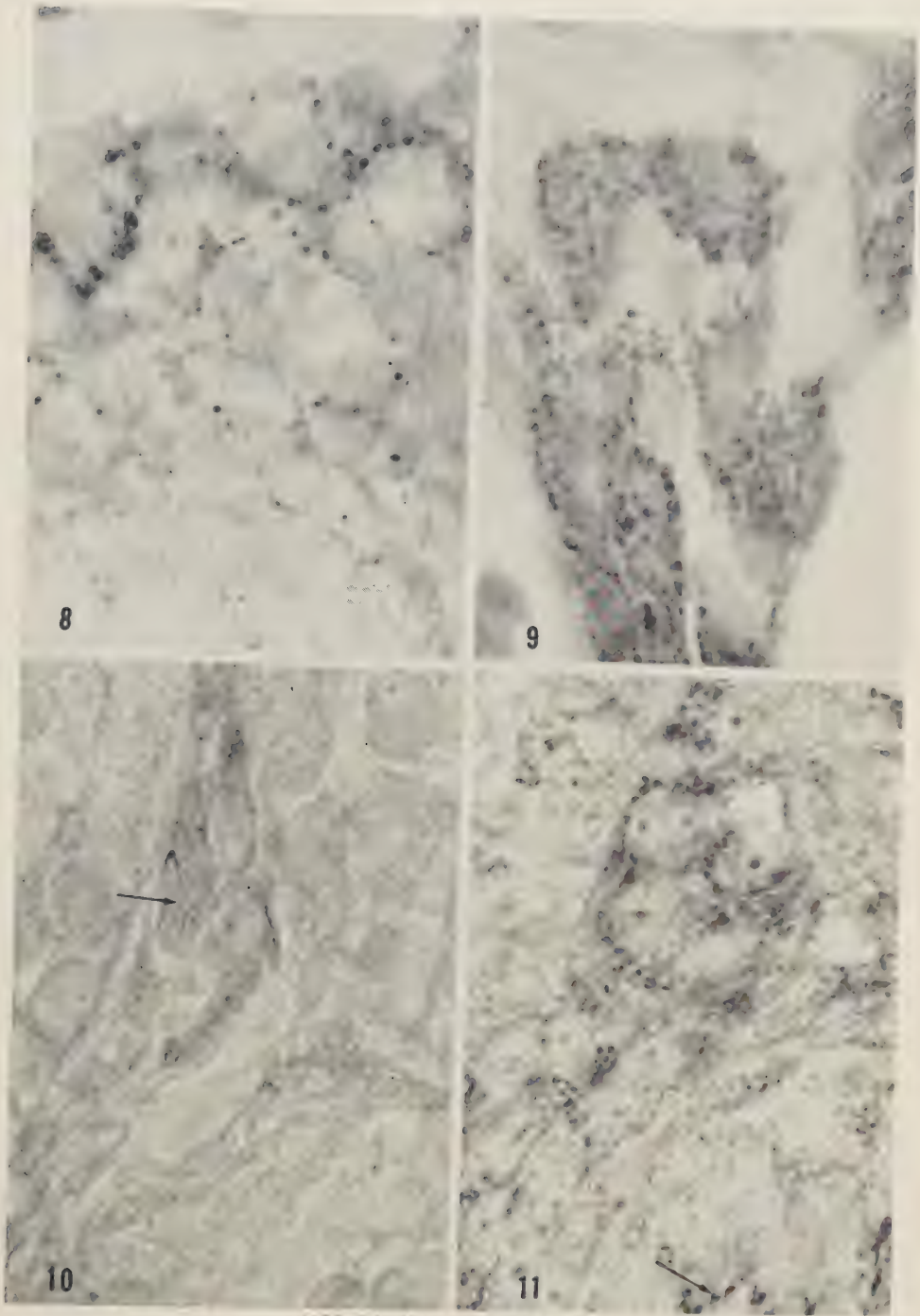


Fig. 4, 6, 7, ×90 objective; ×7.5 ocular. Fig. 5, ×90 objective; ×5 ocular.

FIG. 4. Lipid granules in cytoplasm of Leydig cells of rat testis as demonstrated by Sudan black B technic. Nuclei and nucleoli are unstained (arrow). Similar cell types show variability in reaction. FIG. 5. Seligman and Ashbel method applied to rat testis. Reactive granules in the Leydig cell cytoplasm and unstained nuclei and nucleoli. FIG. 6. Sudan black B-reactive substance in cytoplasm of interstitial cells of guinea pig testis. Differential response in cells containing diffuse, fine, and large granules of sudanophilic material. FIG. 7. The Seligman and Ashbel method applied to testis of guinea pig. Reactive droplets present in the interstitial cells, whereas nuclei and cells within the seminiferous tubule remain unstained.



× 90 objective and × 7.5 ocular

FIG. 8. Sudan black B staining in germ cells of horned lizard. Reactive granules in the cytoplasm of cells. Nuclei and nucleoli are unstained. FIG. 9. Reactive lipid granules in the cytoplasm of Leydig cells of horned lizard as demonstrated by oil red O. Nuclei are counterstained with Mayer's hemalum. FIG. 10. Arrow indicates reactive material in cytoplasm of horned lizard Leydig cells following Seligman and Ashbel method. Nuclei and nucleoli unstained. FIG. 11. Localization of phospholipid in frog testis following Baker's acid hematin test. Reactive lipid in Leydig cell cytoplasm, germ cells, and in nucleoli. Arrow indicates reaction in frog spermatozoon middle-piece.

active granules. A weak response was obtained in these cells in boar and lizard testes. In the bull, ram, boar, and lizard, a stronger reaction was observed in the region of the Sertoli cell cytoplasm which contained embedded spermatozoa. The spermatogonia, primary and secondary spermatocytes, as well as spermatids of the guinea pig and frog showed phospholipid present as fine cytoplasmic granules. Amphibian spermatozoon was unstained except for a small amount of lipid at the anterior tip of the head and in the middle-piece (Fig. 11). The cytoplasm of the spermatogonia, spermatocytes, and spermatids of the other species was weakly reactive.

Cholesterol. A reaction was observed in the Leydig cells of the bull, boar, ram, and guinea pig, rat, horned lizard, and frog. In the individuals examined, the amount of cholesterol in the Leydig cells was variable and in many cases these cells were unreactive.

Polarization microscopy. Birefringent digitonides were present in the cytoplasm of the interstitial and Sertoli cells as well as in the basement membrane of the seminiferous tubules of the frog. Testicular tissue from all other species examined was non-reactive.

Discussion. Intratubular lipids have been described in different animals including the dog, cat, bat, horse, rabbit, and rat(8); the chicken(9); the ram(10); and in man, guinea pig, and mouse by McEnery and Nelson(11). Montagna and Hamilton(12) conducted an investigation dealing with the lipids in the human testis and reported that nearly all of the germinal cells, with the exception of the transforming spermatids, contained fine lipid granules. In addition, large lipid droplets were usually amassed at the periphery of the tubule and occurred in the cytoplasm of some spermatogonia, in nearly all of the peripheral primary spermatocytes, and in the Sertoli cells. According to Montagna and Hamilton(12), most authors believe that the only intratubular lipids present are in the Sertoli cells. However, intratubular lipids were observed in all of the cell types, including the spermatids, in all species studied in the present investigation.

A positive reaction for carbonyl groups was found in the Leydig cells of all species examined, except in the case of the boar, rooster, and fish (Fig. 5, 7, and 10). Ashbel, Cohen, and Seligman(13) studied the testes of frogs, roosters, rats, pigs, and other animals. It is assumed that ketosteroids are responsible in major part for this staining reaction when it is present in tissues in which material possessing the solubility properties of the steroids is found and in which ketosteroids are known to be present(5). In general, the observations made during this investigation are in agreement with those reported for the rat, the rooster, and the frog(13). Ashbel, Cohen, and Seligman(13) reported an intense reaction for ketosteroid in the Leydig cells and a strong one in the Sertoli cells of 6-week-old pigs. It is suggested that the age differential between the young animals used by these investigators and the mature fertile boar used in the work reported here may account for the discrepancy in results obtained with the same procedure.

The presence of phospholipid in the Leydig cells as well as in the middle-piece of the spermatozoon of the mouse has been reported by Baker(7). These results are comparable to those observed in the same cells in the rat in the present investigation. In addition, a positive phospholipid reaction was noted in the Leydig, Sertoli, and germ cells of all species studied with the exception of the bluegill. This procedure was not applied to this species. The heaviest concentrations of phospholipid were observed in the Leydig cells of the horned lizard and of the frog. Fig. 11 illustrates the localization of phospholipid material in the cytoplasm and nucleoli of the interstitial cells and the sperm middle-piece of the frog. It is of interest that phospholipids were localized in all testicular cell types of the species studied in the present investigation. As in the case of the Sudan dye reactions, the phospholipids were variable in amount among the various species as well as between individuals of the same species. However, this substance is considered to play an important role in the metabolic function of the cells of the testis.

A positive Schultz reaction was observed in the interstitial cells of all species studied with the exception of the chicken and fish testes. In view of the question which exists concerning the presence of functional Leydig cells in the fowl and the fish, it is of interest that a

positive reaction was not noted in the testicular interstitium of these species. However, it is possible that if the cyclic breeding season of the bluegill is considered, the specimens employed in this study may have been at a stage during which a reactive quantity of testis cholesterol was absent. A few birefringent digitonides were noted in the Leydig cells and within the seminiferous tubules of the frog testis. In other cases, the dust-like birefringent material observed in the case of the ram, the guinea pig, and the chicken was comparable to that shown by control slides which had been previously treated with acetone. According to Deane and Seligman(14), treatment with acetone at room temperature will dissolve pure steroids. Pollock(15) used the digitonin reaction followed by polarization microscopy and reported that in cat testes, as well as those of other animals, compounds with the chemical properties of testosterone were localized exclusively in the interstitial cells and not present elsewhere in the gland. The importance of the testis in steroid metabolism is further illustrated by the work of Huggins and Moulder(16) in which it was demonstrated that feminizing tumors of the testis were rich in lipids and were growths of Sertoli cells. Likewise, Ashbel, Cohen, and Seligman(13) reported histochemical localization of estrogen in Sertoli cell tumors and in normal cells. Maddock and Nelson(17) have presented evidence that the Leydig cells produce estrogen.

Summary. 1. Comparative histochemical study of vertebrate testes was carried out on sexually mature males representing the classes Mammalia, Aves, Reptilia, Amphibia, and Pisces. Lipids were examined as to their morphological localization in the bull, ram, boar, rat, guinea pig, rooster, the horned lizard, *Phrynosoma cornutum* (Harlan), the frog, *Rana pipiens,* and the bluegill fish, *Lepomis macrochirus* Rafinesque. 2. Following the use of Sudan dyes, lipids were observed in the interstitial cell cytoplasm, the basement membrane, and the cytoplasm of the Sertoli cells, spermatogonia, spermatocytes, and spermatids of all species studied with the exception of the bluegill fish. In the bluegill, lipids were visible in the germ cells and in the interlobular connective tissue sheath. A reaction for carbonyl groups was obtained suggesting the presence of ketosteroid material in the Leydig cells of all species examined, except in the case of the boar, rooster, and fish. With the aid of Baker's acid hematin test, phospholipid was found in the testicular cell types of the species studied. This method was not applied to the bluegill fish testis. Schultz-positive material was noted in the Leydig cells of the bull, ram, boar, rat, guinea pig, horned lizard, and frog. Birefringent digitonides were present in the Leydig cells and seminiferous tubules of the frog. 3. The similarity in the patterns of lipid distribution in the testis of closely related vertebrate species, and those species which are more distant in taxonomic relationship, suggests the structural and metabolic importance of lipids in spermatogenesis as well as in the function of Sertoli and interstitial cells.

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Influence of Cortisone, Piromen and ACTH on Susceptibility of Embryonated Eggs to Fowl-Pox Virus. (21364)

WILLIAM F. HILL AND MELVIN S. RHEINS. (Introduced by Matt. C. Dodd.)

From the Department of Bacteriology, Ohio State University, Columbus.

The influence of cortisone upon the susceptibility of various hosts to experimental viral infections has received considerable attention in recent years. An increase in susceptibility to the infectious agent has been the most frequent observation(1,2,3,4). Generally, the viruses employed in such studies exhibited tissue affinities other than marked dermatropism. The known physiological activities of cortisone and ACTH in delaying or suppressing the inflammatory response(5,6,7,8), depending upon dosage, time interval and route of administration, suggested that parasitism by a dermatropic virus might possibly be altered by treating the susceptible tissue with these agents.

On the other hand, the influence of Piromen, a pyrogenic polysaccharide of bacterial origin, on viral parasitism has received relatively little attention. However, investigations of the pharmacodynamic activity of Piromen have indicated that the pyrogenic agent could potentially benefit the host under conditions of stress induced by viral infections(9,10,11,12, 13,14,15). Consequently, a study to examine the influence and interrelationship of cortisone, Piromen and ACTH upon the susceptibility of the embryonated egg to parasitism by the fowl-pox virus was undertaken.

Materials and methods. Twelve day embryonated eggs were inoculated with the test agents by either the chorioallantoic or allantoic cavity routes while the viral suspensions were introduced onto the chorioallantois. Serial 10-fold dilutions of stock virus suspensions were prepared in chilled nutrient broth. For purposes of titration, each of 4 eggs in a group was inoculated with 0.1 ml of a given viral dilution, incubated at 37°C for 96 hours and examined for evidences of infection. The method of calculating 50% infectivity (EID_{50}) as proposed by Reed and Muench (16) was used throughout the investigation. The infectivity score, determined by the observation of specific macroscopic lesions on the chorioallantois, was facilitated by introducing a sufficient quantity of stain, such as crystal violet, beneath the chorioallantoic membrane by way of the natural air sac. The *cortisone* (Cortone Acetate) used throughout the study was produced by the Merck Co., Rahway, N. J., in the form of a sterile suspension containing 25 mg of cortisone acetate in each ml of isotonic saline solution. The ACTH employed was marketed by The Armour Laboratories, Armour and Co., Chicago, Ill., under the trade name of "Corticotropin ACTH." The Piromen preparations were obtained through the courtesy of the Baxter Laboratories, Morton Grove, Ill., and consisted of commercial preparations (P-70 and P-76) and P-73X, a sample similar to the above except merthiolate was omitted as a preservative. All of the Piromen samples contain the pyrogenic polysaccharide in concentrations of 10 μg per ml, diluted in M/5 Na-r-lactate buffer. A special preparation of Piromen containing 400 μg per ml was also supplied. Control eggs

TABLE I. Infectivity of Fowl-Pox Virus for Eggs Treated with Cortisone by the Chorioallantoic and Allantoic Cavity Routes.

Treatment of eggs	Time interval, hr*	Chorioallantois		Allantoic cavity	
		Test EID_{50}	Control EID_{50}	Test EID_{50}	Control EID_{50}
1.25 mg C† .05 ml NaCl	6	$10^{-6.83}$	$10^{-6.5}$	—	—
1.25 mg C .05 ml NaCl	4	$10^{-5.0}$	$10^{-6.0}$	$10^{-5.85}$	$10^{-5.83}$
1.25 mg C .05 ml NaCl	2	$10^{-4.74}$	$10^{-6.0}$	$10^{-3.83}$	$10^{-5.76}$
1.25 mg C .05 ml NaCl	0	$10^{-4.66}$	$10^{-6.33}$	$10^{-6.16}$	$10^{-6.33}$
1.25 mg C .05 ml NaCl	−2	$10^{-5.22}$	$10^{-5.72}$	$10^{-5.16}$	$10^{-5.5}$
1.25 mg C .05 ml NaCl	−4	$10^{-5.5}$	$10^{-6.0}$	$10^{-5.24}$	$10^{-5.33}$

* Positive numbers indicate time of pre-treatment. Negative numbers indicate time of post-treatment.

† C = Cortisone.

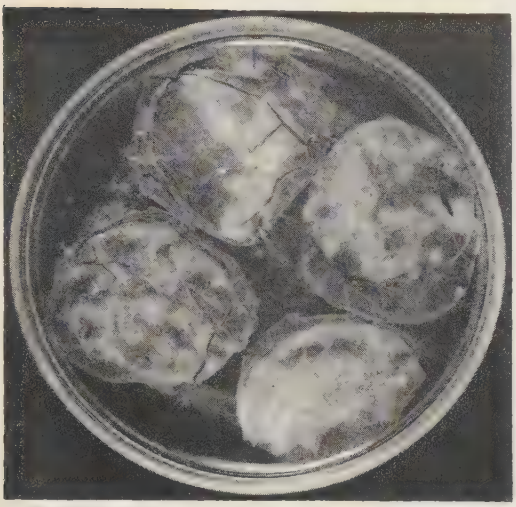

FIG. 1. Chorioallantoic membranes infected with a 10^{-4} dilution of fowl-pox virus.

were inoculated with equivalent volumes of phosphate-buffered saline (pH 7.20).

Results. 1. *The influence of cortisone on fowl-pox infectivity in embryonated eggs.* Embryonated eggs were either pre- or post-treated with 1.25 mg of cortisone by the chorioallantoic and allantoic cavity routes except at the time interval designated as "0 hours" when the virus and cortisone were introduced into eggs simultaneously. In order to ascertain whether or not cortisone exerted a direct effect on the infectivity of the fowl-pox virus, 1.25 mg of cortisone was added to each ml of previously diluted virus. The control sample of virus was diluted in nutrient broth. Both series of tubes were incubated at room temperature for 2 hours before inoculation. The results of treating eggs with cortisone (Table I) indicated that pre-treatment of eggs by the chorioallantoic route at 4 and 2 hours with cortisone, as well as simultaneous inoculation of cortisone and virus, resulted in a significant difference in EID_{50} between the treated and control eggs. However, in the case of the allantoic cavity route only eggs pre-treated at 2 hours showed a marked difference in EID_{50}. A consistent reduction in the size of the viral lesions on the chorioallantois was observed in all the treated eggs whether pre- or post-treated with the hormone and regardless of the route. These differences may be seen by comparing the photographic reproductions (Fig. 1 and 2). Histologic sections of the chorioallantoic lesions from cortisone-treated and control eggs were examined also. A detailed description of the differences in the development of the lesions is being prepared. Results of the study in which cortisone was added to previously diluted virus failed to demonstrate a significant difference in EID_{50}; nor was there a reduction in the size of lesions between the treated and control eggs, indicating that the probable effect of cortisone stems from an altered tissue response rather than from some effect on the fowl-pox virus *per se*.

2. *Influence of Piromen and ACTH on fowl-pox infectivity in embryonated eggs.* Embryonated eggs were pre- and post-treated with various concentrations of Piromen in a manner similar to that described in the study involving cortisone. A multiple dosage titration was also performed. The results indicated that Piromen fails to affect the infectivity of the fowl-pox virus for embryonated eggs. Moreover, no reduction of lesion size was observed with any of the titrations performed. Similar results were obtained in the study in which Piromen was substituted for nutrient broth as the diluent in preparing the virus inocula. Likewise, the treatment of eggs with ACTH, under conditions of this study, had no effect on virus infectivity as measured by

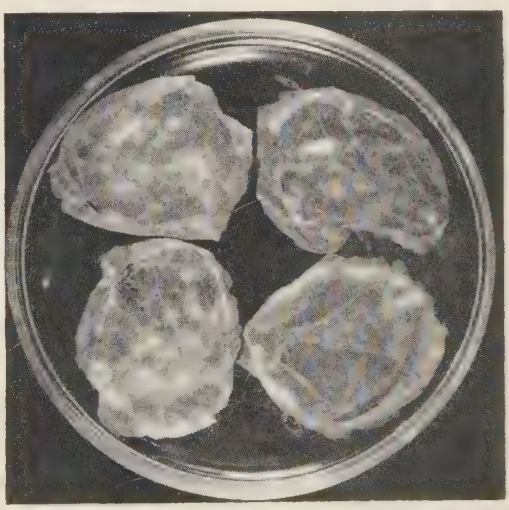

FIG. 2. Chorioallantoic membranes infected with a 10^{-4} dilution of fowl-pox virus. Eggs were post-treated 2 hr with 1.25 mg cortisone onto chorio-allantois.

EID_{50} determinations and by the size of the virus lesions.

3. *Neutralization of effects of cortisone by Piromen and ACTH.* Embryonated eggs were treated simultaneously with cortisone and with Piromen or ACTH as previously described, but only at a time interval at which cortisone alone exhibited an effect on the susceptibility of the eggs as measured by EID_{50}. The results of this study indicated that a reversal of the observed influences of cortisone was demonstrated by the simultaneous inoculation of Piromen or ACTH with this hormone, suggesting a similarity of action of Piromen and ACTH in this respect.

Discussion. The mode of action by which cortisone increases the resistance of the chorioallantois to infection with the fowl-pox virus is not understood at present, but probably involves a variety of local tissue changes which already have been attributed to cortisone. Likewise, the antagonism of these effects by Piromen and ACTH requires further investigation, but the immediacy of the antagonism suggests a local inhibition rather than a more remote systemic influence.

Summary and Conclusions. 1. The pre- and post-treatment of embryonated eggs with 1.25 mg of cortisone by the chorioallantoic and allantoic cavity routes significantly decreased the susceptibility of the chorioallantois to infection with the fowl-pox virus as measured by EID_{50} determinations and marked reduction in development of virus lesions. However, the incubation of diluted virus with cortisone (1.25 mg/ml), prior to inoculation onto the chorioallantois, did not alter the EID_{50}, nor was the development of the virus lesions impaired. 2. The pre- and post-treatment of embryonated eggs with various concentrations of Piromen or constant quantities of ACTH (1.25 mg) by the chorioallantoic and allantoic cavity routes failed to alter the susceptibility of the chorioallantois to infection with the fowl-pox virus. Likewise, the incubation of Piromen with virus at room temperature failed to alter the infectivity of the fowl-pox virus. 3. Simultaneous treatment of eggs with cortisone and Piromen or ACTH resulted in inhibition of the effects observed with cortisone alone. Moreover, the reduction in lesion size, although discernible, was not as great as with cortisone.

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Effect of Acute Hepatic Ischemia on Splanchnic Hemodynamics and on BSP Removal by Liver.* (21365)

Ewald E. Selkurt.

From Department of Physiology, Western Reserve University School of Medicine, Cleveland, O.

Increasing attention is being directed to consequences of hepatic anoxia on physiological mechanisms of shock. The deleterious influences of impaired hepatic circulation culminating in hepatic coma have been recently described by Rappaport *et al.*(1) and by Giges *et al.*(2). They have fully documented the metabolic consequences, but information concerning hemodynamic sequellae of hepatic ischemia have apparently not yet been fully described. The purpose of the present investigation was to study the effects on splanchnic circulation of acute periods of liver ischemia lasting 1 to 2 hours. The BSP method for estimating hepatic venous outflow was employed to assess the hepatic circulation, and concurrent observations were made on the effect of anoxia on liver's ability to remove BSP from plasma.

Method. Dogs averaging 19 kg were anesthetized with pentobarbital sodium, 30 mg per kg I. V. A mid-line abdominal incision was made followed by splenectomy and placement of loose ligatures around portal vein and hepatic artery near the liver. The splenic vein was cannulated and connected by Tygon tubing to a sound introduced into the superior vena cava via an external jugular vein. This connection served as a shunt to allow drainage of mesenteric vascular channels during hepatic ischemia. A saline manometer connected to the splenic cannula measured mean portal venous pressure. The level of the inferior vena cava was the reference point for the zero pressure of this manometer. Mean arterial blood pressure was continuously measured with a mercury manometer from a carotid artery. Following a 50 mg priming dose, bromsulphalein was continuously infused intravenously with a calibrated motor-driven syringe at average rate of 0.047 mg/kg/min. (range, 0.039-0.051) (Table I). A 30-minute equilibration preceded a 30-minute control period. The infusion was then stopped, the animals were heparinized, and the hepatic artery and portal vein were occluded by pulling the vessels snugly against ends of glass tubing through which ligatures had been passed. Simultaneously with closure of the portal vein, the shunt circuit was opened. *Hepatic ischemia* was continued 1 hr, 1½ hr, or 2 hr periods. Upon release of occluding ligatures, the shunt circuit was closed, and flow reestablished through the liver. A small priming dose of BSP was usually given, and dye infusion was reinstituted and continued for about 90 minutes post-ischemia. *Blood* for dye analysis was drawn simultaneously from a femoral artery and from a hepatic vein via a metal sound which was introduced through an external jugular vein. Cannulae were flushed prior to taking blood samples. Samples were taken at 0, 10, 20, and 30 minutes of control period, and at 10, 20, 30, 45, 60, 75, and 90 minutes following release of ligatures. The blood was rapidly centrifuged and plasma removed for dye analysis; the cells were suspended in saline, and returned to the animal. Variable amounts of blood were obtained by draining the excised spleens; this served as an additional source of blood to aid in maintaining blood volume during the experiment. This was further supplemented with donor blood to make up the deficit incurred by blood removal for dye analysis and for hematocrit determinations, and that lost by oozing from incisions. Chemical methods employed for BSP analysis have been previously described(3,4). *Arterial blood pressure* was reasonably stable during the shorter periods of hepatic ischemia (1, 1½ hours). When decreases in blood pressure occurred, at times during the 2 hours ischemia, stabilization was obtained by injections of glucose in saline and small supplementary blood transfusions.

* This investigation was supported by research grant from the National Heart Institute, National Institutes of Health, U. S. Public Health Service, Washington, D.C.

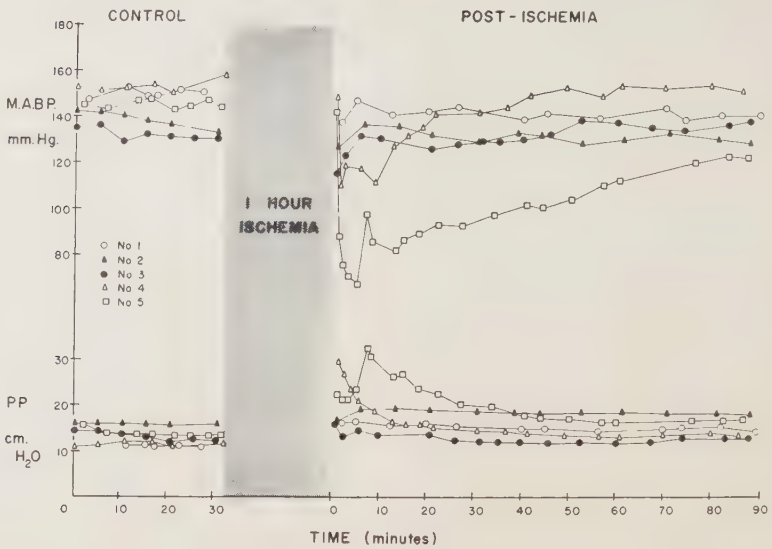

FIG. 1. Effect of 1 hr hepatic ischemia on mean arterial blood pressure (M.A.B.P.) and mean portal venous pressure (P.P.).

Results. 1. *Effect of hepatic ischemia on arterial and portal venous pressure.* Ischemia of one hour's duration caused no significant effect on arterial blood pressure in experiments No. 1, 2, 3. Portal venous pressure showed only minimal increases (10.2 to 16.5, 15.7 to 18.8, and 12.0 to 14.0 cm saline, respectively) (Fig. 1). In Exp. 4, blood pressure decreased from 148 to 110 mm Hg one minute after release of ligatures, and portal pressure increased rapidly from 12.0 to 29.3 cm. In this animal, compensation was rapid, and arterial pressure was restored to 141 mm Hg in 21 minutes, as portal pressure declined to 15 cm. Exp. 5 showed a more drastic response. Four minutes after release of ligatures, arterial blood pressure had decreased from 140 to 67 mm Hg, while portal venous pressure rose to 32.3

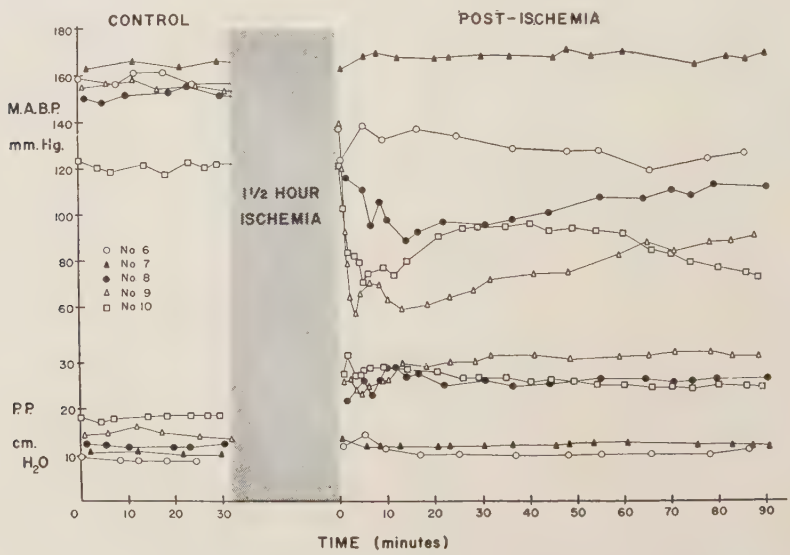

FIG. 2. Effect of 1½ hr hepatic ischemia on mean arterial blood pressure and portal venous pressure.

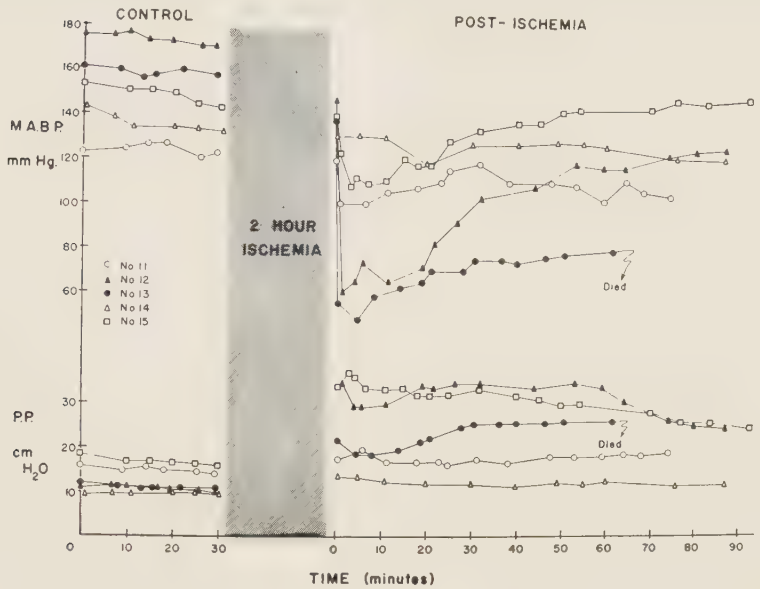

FIG. 3. Effect of 2 hr hepatic ischemia on arterial pressure and portal venous pressure.

cm. However compensatory mechanisms operated to restore arterial pressure to 121 mm Hg 86 minutes post-ischemia.

Three of the 5 animals showed significant hemodynamic alterations following 1½ hours' ischemia (Fig. 2). In these (Exp. 8, 9, 10) blood pressure fell sharply to 88, 56, and 70 mm Hg respectively following release of hepatic ligatures, with concomitant increases in portal venous pressure to a range of 25 to 30 cm saline. In these animals, portal pressure remained elevated during the 90 minutes of observation, and arterial pressure was only partially restored. In Exps. 6 and 7 no significant alterations in arterial blood pressure were noted, and the portal pressure showed no noteworthy changes.

Four of the animals in the group subjected to 2 hours of ischemia showed typical response following hepatic ischemia, *viz.*, a rapid decline in arterial pressure and simultaneous elevation in portal pressure (Fig. 3). However, responses were somewhat variable. In Exp. 11 arterial pressure changed only from 119 to 100 mm Hg, while portal pressure increased from 14 to 18 cm saline. At the other extreme, Exp. 12, pressure collapsed from 147 to 60 mm Hg as portal venous pressure rose from 10 to 34 cm. Three animals exhibited a considerable degree of compensation of the arterial pressure, but one (No. 13) did not, and died abruptly 75 minutes after reestablishment of hepatic circulation. As in earlier series, portal pressure was characteristically elevated in those animals which exhibited a decline in arterial pressure. One animal of this series (Exp. 14) showed no significant response to hepatic ischemia of this duration.

2. *Effect of hepatic ischemia on BSP removal.* Results are given for all animals except No. 15. In this experiment, infusion of BSP was purposely reduced after ischemia, but was not sufficient to attain plasma levels suitable for analytical accuracy, and results are therefore not included in Table I.

Exp. 1, 2, and 3 were characterized by actual increases in the extraction ratio to 82, 46, and 67% above control, probably the result of reduced hepatic blood flow permitting more time for dye removal. The arterial plasma concentration remained rather constant. In Exp. 4 and 5 the ratio was respectively 54 and 97% of the control, and plasma concentration of the dye increased progressively during the post-ischemic period.

Animals in the 2nd series showed a reduction in the BSP extraction ratio with progressive increase in arterial concentration. This

TABLE I. Effect of Ischemia on BSP Removal and EHBF.

	Control					Post-ischemia									
								Arterial conc.					Exp.		Exp.
Exp.	Wt, kg	Infusion, mg/min. /kg	Arterial conc., mg %	Extraction	EHBF, ml/min.	Prime 1 hr ischemia	Infusion, mg/min. /kg	10 min.	90 min.	Avg†	% incr.‡	Extraction	Control	EHBF,§ ml/min.	Control
1	12.5	.049	1.29‖ (1.24-1.36)	.227‖ (.191-.267)	457‖ (376- 563)	20	.047	1.24	1.19	1.15	−4	.413‖ (.376-.469)	1.82	234‖ (204-252)	.51
2	24.0	.039	.88 (.88- .89)	.218 (.187-.255)	949 (801-1092)	30	.039	1.46	1.49	1.40	2	.318 (.281-.368)	1.46	411 (337-451)	.44
3	21.6	.047	.85 (.79- .89)	.212 (.178-.229)	1229 (1090-1410)	20	.047	1.04	1.16	1.03	12	.353 (.324-.382)	1.67	585 (455-634)	.48
4	16.5	.048	1.10 (.94-1.20)	.374 (.330-.411)	413 (342- 566)	10	.046	2.35	3.19	2.80	36	.200 (.108-.278)	.54	———	—
5	14.2	.047	.96 (.91- .98)	.107 (.094-.123)	1386 (1128-1640)	5	.047	1.72	4.03	2.89	134	.104 (.053-.152)	.97	364 (255-534)	.26
						1½ hr ischemia									
6	20.0	.046	1.10 (1.03-1.19)	.220 (.175-.265)	721 (570- 823)	15	.046	.82	1.25	1.19	53	.187 (.069-.261)	.85	649¶ (361-765)	.90
7	25.0	.051	1.57 (1.45-1.65)	.260 (.224-.296)	845 (680-1000)	20	.051	1.62	2.04	1.73	26	.250 (.120-.383)	.96	648¶ (540-853)	.77
8	13.4	.047	.75 (.73- .76)	.322 (.283-.343)	500 (439- 552)	15	.047	2.58	4.25	3.31	64	.079 (.056-.100)	.25	———	—
9	23.5	.052	1.01 (.98-1.09)	.305 (.276-.348)	841 (677- 950)	10	.055	2.56	5.35	4.21	110	.128 (−.035-.266)	.36	———	—
10	22.3	.045	.65 (.62- .68)	.183 (.137-.207)	1747 (1440-2360)	—	.023	1.06	1.72	1.40	63	.082 (.019-.145)	.45	———	—
						2 hr ischemia									
11	13.5	.045	.60 (.59- .60)	.464 (.416-.500)	437 (400- 481)	—	.045	.52	.97	.78	87	.293 (.163-.378)	.63	443¶ (392-484)	1.01
12	20.5	.049	1.00 (.88-1.10)	.490 (.446-.530)	467 (397- 554)	15	.049	2.98	4.59	4.04	54	.067 (.013-.127)	.14	———	—
13	19.0	.045	.80 (.77- .82)	.261 (.214-.299)	944 (782-1150)	—	.028	1.40	3.26	2.32	133	.071 (−.007-.118)	.27	———	—
14	18.5	.046	1.31 (1.29-1.38)	.486 (.455-.510)	270 (260- 294)	—	.013	.35	.62	.44	77	.120 (.000-.212)	.25	———	—

* Avg of 4 observations except Exp. 10 and 11 in which only 3 control observations are included.
† Avg of usually 7 observations taken during post-ischemic period. In Exp. 5 and 11 only 6 observations were made, and in Exp. 13 only 5.
‡ The % increase signifies change in concentration from first (10 min.) concentration to the last (usually 90 min. except for Exp. cited above).
§ Calculated only for those experiments in which the extraction ratio averaged at least 63% of control during post-ischemia.
‖ Avg and (range).
¶ In these experiments EHBF could not be calculated at times when the extraction was zero, or nearly so. This occurred once in each experiment.

increase was not great in Exp. 6 and 7, which exhibited minimal hemodynamic alterations. In Exp. 8, 9, and 10 marked reduction in the liver's ability to remove the dye was indicated by the large decreases in the extraction ratio and by increases in dye concentration to 63-110% above the initial post-ischemic concentration during the 1½ hours' observation following release of the hepatic ligatures.

All showed marked reduction in BSP extraction, and dye concentration increased following ischemia of 2 hours' duration to 54-133% above the initial value. Complete failure of extraction or nearly so was noted occasionally in Exp. 12, 13, and 14.

3. *Effect of ischemia on EHBF.* Effective hepatic blood flow (EHBF) was computed according to the method of Bradley *et al.*(5), with appropriate corrections for changing arterial dye concentrations. It should be mentioned that reservations must be entertained concerning the validity of this method for estimating hepatic blood flow under circumstances of possible liver damage and impaired ability to remove BSP, as found after severe ischemia. It is emphasized that calculated EHBF values may not necessarily represent actual hepatic venous outflow, particularly in those experiments where BSP extraction is demonstrably reduced. Recognizing this fact, the results presented in Table I have been selected from those experiments which show average BSP extraction ratios of not less than 0.63 of control following ischemia. At best, these data can only supply inferential information regarding the direction of change of hepatic blood flow, since with impaired extraction the nature of the calculation would yield erroneously high blood flow figures. Despite these reservations, the data of Table I favor the conclusion that hepatic blood flow is reduced following hepatic ischemia of one hour's duration or longer.

Discussion. The significant finding in this investigation was the collapse in arterial blood pressure and concomitant increase in portal venous pressure which frequently followed the restoration of hepatic circulation after ischemia and to which the animals compensated to varying degrees during 1½ hours' observation. The present series was not observed for ultimate survival. Explanation of basic mechanisms can at present only be inferential, but a suggestive clue is the increased portal pressure. This could result from (a) increased hepatic vascular resistance relative to mesenteric vascular resistance; (b) reduced mesenteric resistance relative to hepatic resistance; and (c) combinations of increased hepatic resistance and reduced mesenteric resistance.

In the absence of a cardiac factor†, the decrease in blood pressure must result from vascular dilatation in which the mesenteric vascular bed would be expected to participate, based on the rise in portal venous pressure. The possibility that increased hepatic resistance contributes to the rise in portal pressure should also be entertained, particularly since the data on EHBF suggest a reduced liver blood flow. But this could be expected with the observed fall in blood pressure which occurred in most cases. Because of doubt regarding the quantitative validity of the hepatic blood flow measurements, calculation of hepatic vascular resistance was not attempted, and a final decision cannot be made on this point.

The hemodynamic observations are in conformity with the hypothesis that when the liver is made anoxic and then the circulation is reestablished a substance (or substances) is released into the circulation which often results in precipitous circulatory collapse probably involving dilatation of the mesenteric vasculature. A better understanding of the nature of this substance, and the mechanism of its action (directly on peripheral vessels or centrally via the vasomotor centers) awaits further experimentation.

The present investigation raises the question of the validity of the BSP method for measuring liver blood flow under conditions where the liver is damaged. The current data on BSP extraction suggest that the hope for quantitative estimates appears to become increasingly uncertain beyond an hour's ische-

† In recent experiments conducted with optical registration of arterial pulse, cardiac slowing at times accompanied the fall in arterial pressure. However, blood pressure alterations occurred often without significant alteration in heart rate so that it can be considered only a contributory factor.

mia. In dogs surviving hepatic artery and portal vein closure because of development of collateral blood supply, the liver's loss of ability to remove BSP has been largely attributed to the reduced hepatic blood flow(6). Hepatocellular function (for those parenchymal cells which receive blood) appears to be nearly normal. The rising plasma concentrations in the present experiments following ischemia could result from combinations of reduced blood flow and impaired extraction.

Although the magnitude of the observed responses were largely related to the duration of the ischemia, exceptions occurred which raise the question of the reason for the individual variability. Two probable explanations are offered; (1) variation in the nutritional state and physical condition of the individual animal; (2) establishment of varying degrees of collateral blood supply during portal vein and hepatic artery closure, which would obviously reduce the severity of the anoxia.

Summary. 1. Hepatic ischemia was produced by closure of the hepatic artery and portal vein of dogs for periods of 1 to 2 hours. This caused, in the majority of instances, a rapid and often profound decrease in mean arterial blood pressure, with concomitant sharp increase in portal venous pressure, following restoration of blood flow through the anoxic liver. The animals were usually able to compensate with partial to complete restoration of blood pressure, and simultaneously some degree of reduction of portal pressure during 1½ hours of observation following ischemia. However one animal (1½ hour ischemia) entered a declining phase of arterial pressure one hour after ischemia, and a second animal (2 hour ischemia) died 75 minutes after ischemia. Possible mechanisms for the hemodynamic alterations are discussed. 2. BSP extraction was reduced in only one of 5 experiments in which ischemia was of one hour's duration. However, extraction was usually markedly reduced following 1½ to 2 hours of ischemia, with zero extraction or nearly so being a common finding in individual arterio-venous comparisons.

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Effect of Lipemia and Heparin on Free Fatty Acid Content of Rat Plasma.* (21366)

MORTON I. GROSSMAN, LUCILLE PALM, GERALD H. BECKER, AND HUGO C. MOELLER.
From the Medical Nutrition Laboratory, Fitzsimons Army Hospital, Denver, Colo.

Nichols *et al.*(1-3) have reported that during *in vitro* clearing of egg lipoprotein by post-heparin plasma, free fatty acid is released. We(4) have confirmed this finding in an *in vitro* system consisting of post-heparin plasma and coconut oil emulsion (Fig. 1). The present study was undertaken to determine whether heparin-induced lipemia clearing *in vivo* is accompanied by a rise in free fatty acid content of the plasma.

Methods. Male albino rats weighing between 160 and 225 g were used. After an overnight fast the following treatments were given: Group A, no treatment, fasting controls; Group B, 2 ml of corn oil was given by stomach tube and blood was drawn 3 hours later; Group C, 10 mg/kg of heparin was injected intravenously and blood was drawn 15 to 20 minutes later; Group D, 2 ml of corn

* The opinions expressed in this paper are those of the authors and do not necessarily represent those of any governmental agency.

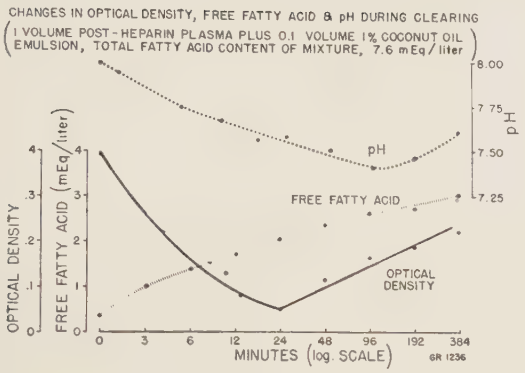

FIG. 1.

oil was given by stomach tube, 3 hours later 10 mg/kg of heparin was injected intravenously and blood was drawn 10 to 20 minutes later. Under light ether anesthesia, blood was drawn from the aorta in chilled syringes and kept at 5°C or less throughout centrifugation and until sampling of the serum or plasma for free and total fatty acid determinations. The free fatty acid was determined by a method devised for this study and based in part on the method described by Davis(5). Two ml of serum or plasma was added to 0.2 ml 0.2 M phosphate buffer (pH 6.0) and 2 ml 95% ethyl alcohol was then added. This mixture was extracted 3 times with 3 ml portions of petroleum ether (B.P. 30-60°C) by shaking for 1 minute, centrifuging, and removing the supernatant with a syringe and needle. The combined petroleum ether extracts were evaporated to dryness in a bath at 70°C and the residue was taken up in 2 ml of alcohol, heated, and titrated with 0.02 *N* aqueous sodium hydroxide, using thymol blue as the indicator. Carbon dioxide-free air was bubbled through the alcohol solution during the titration. Blanks, in which water was used in place of serum, were run with each set of determinations. The results are expressed as milliequivalents of free fatty acid per liter. In 16 samples run in triplicate the standard deviation of a single determination (square root within samples variance) was 0.083 meq/l. The determinations reported under Results were done in duplicate. Total fatty acid was determined by the method of Smith and Kik (6), with slight modification. In 15 samples on which triplicate determinations were done the standard error of a single determination (square root of within sample variance) was 0.303 meq/l. The determinations reported under Results were done in duplicate.

Results. The results are summarized in Table I. The mean value for free fatty acid in the serum of fasting rats was 0.358 meq/l. Injection of heparin into fasting animals or induction of alimentary lipemia led to similar rises in the free fatty acid content, 0.603 and 0.592 meq/l, respectively. In the former case this was associated with no change in total fatty acid, while in the latter there was a marked rise in total fatty acid so that the per cent of fatty acid in the free form rose in the heparin-injected fasting animals and fell in the lipemic animals. Injection of heparin into lipemic animals resulted in the highest values for free fatty acid seen in the 4 groups, 1.763 meq/l. This was accompanied by a fall in total fatty acid (as compared with lipemic animals not receiving heparin) and thus with a marked increase in the per cent of fatty acid in the free form. All differences between group means of free fatty acid values were statistically significant ($P < 0.01$) except that between groups B and C.

Because the release of free fatty acid is known to continue *in vitro* it was necessary to determine whether significant alteration in free fatty acid content occurred between the time the blood was drawn and the time the determination was begun. Four pools of serum or plasma, corresponding to the four treatment groups described above, were prepared. Free fatty acid content of the samples was determined as soon after drawing blood as possible (within 2 hours), and again after storage at room temperature for 4 hours and at 5°C for 4 hours and 24 hours. The results are presented in Table II (mean values of triplicate determinations) and show that no marked change occurred with storage at 5°C for 4 hours but 4 hours at room temperature or 24 hours at 5°C resulted in large increases. These results indicate that under the conditions of this study (blood drawn in chilled syringes and refrigerated until determinations were begun, which was always less than 4

TABLE I. Free and Total Fatty Acid Content of Rat Serum or Plasma after Various Treatments.

Treatment	Free fatty acid (meq./l) N	Mean	S.D.	Total fatty acid (meq./l) N	Mean	S.D.	% of fatty acid in free form N	Mean	S.D.
A. 16 hr fast	21	.358	.114	15	6.25	1.39	14	6.8	1.74
B. 3 hr after 2 ml corn oil intragastrically	21	.592	.160	8	16.27	5.08	8	3.9	1.08
C. 18 min. after 10 mg/kg heparin, intrav.	22	.603	.177	15	5.47	.92	15	11.8	1.84
D. 14 min. after 10 mg/kg heparin, intrav. given 3 hr after corn oil	16	1.763	.785	9	8.81	1.87	9	17.3	3.40

hours after drawing blood) the values represent the true *in vivo* concentration of free fatty acid.

Discussion. Little information is available on free fatty acid content of serum. Davis(5) found 0.4 meq/l in human serum. Cohn *et al.*(7) reported that serum albumin as prepared by them contained as much as 1 mole of fatty acid per mole of albumin. The free fatty acid content of lymph has been more extensively studied. Freeman and Johnson (8) have reviewed the literature on the subject and reported their own values of 0.1 to 0.2 meq/l for fatty chyle from dogs. Reller *et al.*(9) found that free fatty acid constituted an average of 1.1% of the acetone soluble lipid of lipemic rat chyle. Several investigators have shown that serum albumin has a rather high capacity to bind fatty acid. Teresi and Luck(10) found that there is considerable affinity between serum albumin and fatty acid anions, the strength of the non-polar attraction being increased with longer chain length. Gordon *et al.*(11) cite unpublished data of Dr. H. A. Saroff who found that at pH 4.3, one mole of serum albumin can bind 2 moles of palmitate and at pH 6.5 it can bind 6 moles. Davis and Dubos(12) state that one mole of serum albumin can bind 9 moles of oleate, but do not give the pH conditions. Gordon *et al.* (11) reported that oleate inhibits heparin-induced lipemia clearing, an effect which they found to be reversed by albumin. When 7 to 8 moles of oleate per mole of albumin were present, clearing was completely inhibited but little inhibition occurred with lower ratios. Robinson and French(17) found that during *in vitro* clearing of rat chyle by post-heparin rat plasma the fatty acid content of albumin rose to from 5 to 6 moles per mole of albumin.

The concentration of albumin in rat plasma expressed as meq/l is about 0.5. Assuming that 6 moles of fatty acid can be bound by one mole of albumin, about 3.5 meq of fatty acid per liter of plasma could be so bound. All of the values we found were lower than this and, therefore, it may be assumed that most of the fatty acid was albumin bound.

The sharp drop in total fatty acid content of the plasma of lipemic rats after heparin injection indicates that the effect of heparin is not only to change the physico-chemical state of the fat in the blood but also to speed its removal from the blood.

TABLE II.
Free Fatty Acid Content of Rat Serum or Plasma after Storage at Room Temperature or 5°C.

Treatment of animals	Free fatty acid (meq./l) Initial	4 hr room temp.	5°C 4 hr	5°C 24 hr
A. Fasting	.48	.62	.46	.58
B. 3 hr after 2 ml corn oil intragastrically	.74	1.45	.68	1.18
C. 15 min. after 10 mg/kg heparin intrav.	.83	1.37	.76	1.07
D. 15 min. after 10 mg/kg heparin intrav., 3 hr after 2 ml corn oil intragast.	1.27	2.06	1.31	1.39

The role which lipolysis and release of free fatty acid plays in the mechanism of heparin-induced clearing is not clearly established(3, 13). It may be the main mechanism causing the clearing or it may be an associated phenomenon. Since several investigators have presented evidence indicating that heparin-induced clearing is simply an acceleration of a normally occurring process(14-16), the present studies would suggest that lipolysis and free fatty acid release may play an important role in fat metabolism. This hypothesis is supported by the rise in free fatty acid level in the plasma seen with alimentary lipemia without heparin injection.

Summary. Injection of heparin produces a moderate rise in the free fatty acid content of the plasma of fasted rats and a marked rise in lipemic rats. In the latter case this is accompanied by a precipitous fall in total fatty acid content. A moderate rise in free fatty acid content also occurs in association with lipemia without heparin injection.

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Hereditary Differences in Serum Proteins of Normal Mice.* (21367)

SARANE THOMPSON,† JOSEPH F. FOSTER,‡ JOHN W. GOWEN, AND OSCAR E. TAUBER.

From the Departments of Chemistry, Genetics, and Zoology, Iowa State College, Ames.

There have been frequent references in literature to the "normal values" of serum proteins in laboratory animals. When an untreated animal deviated from the norm, it was assumed that the environmental control of the experiment was inadequate. In regard to electrophoretic analyses, it was implied that there were no qualitative serum protein variations among healthy animals of the same species. Moore reported that each of 20 species had a "characteristic and reproducible" electrophoretic pattern(1).

It has recently been shown that normal animals of the same species may vary in amount of total serum protein(2) and in electrophoretic pattern(3,4,5). In the present study, inherent differences in serum proteins have been

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† Present address: Department of Biology, San Francisco College for Women.

‡ Present address: Department of Chemistry, Purdue University.

TABLE I. Total Serum Protein in 7 Mouse Strains (g %).

Strain	S	RI	K	Z	E	LGW	BALB /Gw
Mean*	6.20	5.96	6.19	5.81	6.83	6.20	6.22
S.E.†	.03	.07	.04	.13	.07	.05	.07

* Each mean represents duplicate analyses of 4 samples of pooled sera. Each sample contained the sera of at least 10 female mice.
† Stand. error.

demonstrated in inbred mouse strains. The mode of inheritance of a β_1-globulin has been determined.

Materials and methods. The 7 strains of mice used in this investigation had been highly inbred by over 30 generations of brother-sister matings. As a result, each strain was a homogeneous population. For each plasma protein analysis, the blood of at least 10 mature female mice was pooled. Total serum protein values were obtained from duplicate micro-Kjeldahl determinations. For *electrophoretic analyses,* the pooled serum samples were dialyzed against buffer for at least 2 days at 2°C. The phosphate-chloride buffer was composed of 0.15 M NaCl, 0.0197 M K_2HPO_4 and 0.0012 M KH_2PO_4. The ionic strength was 0.21 and the pH was 7.8. The analyses were made in a Tiselius cell at 2°C at a potential gradient of 4.0 volts/cm. Resolution of the boundaries was attained by use of the Philpot-Svensson optical system. Patterns were enlarged 3 diameters above the dimensions of the cell and traced onto graph paper. Tracings of ascending and descending patterns were placed on one graph. This procedure was repeated and independent interpretations of the 2 graphs were made. Areas were measured by a planimeter. The additional time required for 2 tracings was justified by a statistical analysis which revealed that tracing interpretation error made up about half the experimental error in analyses of normal mouse serum.

Results. In Table I, it may be seen that the strains of mice differed in the concentration of total protein in the serum. When compared with the other 5 strains, the E strain was higher, and the Z strain was lower in total protein. These relationships are statistically significant ($P<0.01$). The mean value for all 7 strains, 6.2 g %, is in agreement with values established for mouse serum by other workers(6,7).

No consistent strain differences in the percentage protein composition of normal serum could be demonstrated. The results of thirty electrophoretic analyses appear in Table II. These values are in accord with those found in similar studies of mouse serum(5,6,8). The coefficients of variation obtained in these analyses of serum from several inbred strains are no higher than those obtained from the serum proteins of a supposedly uniform population(9).

A β_1-globulin peak was observed only in serum of the E strain. This electrophoretic component was invariably found to be present in the 10 samples of pooled E serum which were analyzed. It was never observed in the more than one hundred electrophoretic analyses of serum from the mice of other strains. In normal serum, the concentration of this component was low and its resolution was poor (Pattern 2, Fig. 1). However, in *Salmonella typhimurium* infections, this component increased to twice its normal level. Because the resolution of the β_1-globulins was improved when their concentrations were increased, further studies were made on the serum of infected mice. In Fig. 1, Patterns 3, 4 and 5 were made on serum drawn from animals on the fourth day of an experimental mouse typhoid infection.

Each of the 7 mouse strains used as a source of blood for this investigation has been inbred by brother-sister matings for over 30 generations. In these inbred colonies, the β_1-globulin component has been found only in the E strain. To determine something of the

TABLE II. Electrophoretic Analyses of Thirty Serum Samples.*

Protein	Mean†	Stand. error	Coef. of variation (%)
Albumin	60.8	.7	7
Globulin			
α	12.1	.4	19
β	20.4	.6	16
γ	6.7	.2	18

* Each sample contained pooled sera of at least 10 female mice.
† % of total serum protein.

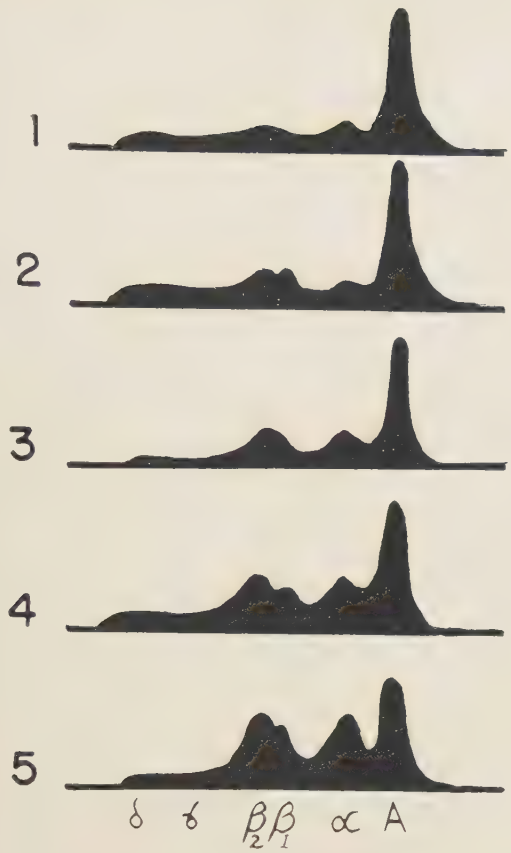

FIG. 1. Ascending electrophoretic patterns of 5 serum samples. Each sample contained pooled sera of at least 10 female mice. Pattern 1. Strain S—normal serum. Pattern 2. Strain E—normal serum. Pattern 3. Strain S—serum from *Salmonella typhimurium* infected mice. Pattern 4. Strain E—serum from *S. typhimurium* infected mice. Pattern 5. Hybrid (S × E)—serum from *S. typhimurium* infected mice.

inheritance pattern of this unusual plasma component, male S mice were mated with females of the E strain, and female S mice were mated with males of the E strain. The hybrid progeny which resulted from these 2 reciprocal crosses were infected with *S. typhimurium*. Such infection does not change the basic electrophoretic pattern of either strain S mice or strain E mice, except to alter the magnitude of some parts of the patterns. (Fig. 1). On the fourth day of the infection, the hybrid E × S mice were sacrificed to obtain four samples of pooled serum: one sample from males and females derived from each of the 2 reciprocal crosses. Each sample contained the serum from at least 10 mice. Electrophoretic analyses revealed that the β_1-globulin component was present in blood from all 4 types of progeny. This seems to indicate that the gene (or genes) which determine the presence of the component are dominant. It is unlikely that the trait is sex-linked.

Summary. In a study of the serum proteins of 7 mouse strains, it was established that there were inherent differences in the electrophoretic patterns and in the amount of total serum protein. A distinct β-globulin component was found in only one, E, of the strains. The hybrid progeny of this strain also had this globulin component in their serum. This indicates that the protein is probably determined by a dominant gene (or genes).

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Evidence for a Role of the Supraopticohypophyseal System in Regulation of Adrenocorticotrophin Secretion. (21368)

S. M. McCann and J. R. Brobeck. (Introduced by F. D. W. Lukens.)

*From the Department of Physiology and the George S. Cox Institute, University of Pennsylvania School of Medicine, Philadelphia.**

A failure of secretion of adrenocorticotrophin (ACTH) follows certain operations upon the hypothalamus in rats, dogs, and cats(1,2, 3), especially when the median eminence of the tuber cinereum is injured. This failure has been demonstrated by direct assays of ACTH levels in blood and the anterior pituitary gland, using for the assay rats with appropriate hypothalamic lesions, subjected to adrenalectomy and then maintained on desoxycorticosterone until the day of sacrifice under acute stress(4). Under these conditions no ACTH can be detected in the blood, while the pituitary level of ACTH is reduced by 50%. The mechanism by which hypothalamic lesions suppress ACTH production and block its release by the pituitary has not been elucidated. Three possibilities exist to explain this effect. First, lesions of the median eminence by injuring the hypophyseal portal system may impair adenohypophyseal circulation to such an extent that the hypoxic, malnourished pituitary can no longer function adequately. This possibility appears unlikely since the gland appears normally vascularized and has a normal cellular structure histologically(1,3). A second possibility is that the effective lesions block a direct secretomotor innervation to the gland. This seems unlikely since stalk section is compatible with normal discharge of ACTH in stress(5,6,7). Lastly, it has been proposed by several workers that a neurohumoral substance is released in the median eminence. This neurohumoral agent traverses the hypophyseal portal vessels and activates the anterior lobe(8,9). Direct evidence for such neurohumoral activation of the anterior lobe has been lacking.

In this communication evidence is presented which suggests that destruction of the supraopticohypophyseal tract is responsible for the blockade of ACTH release in rats with hypothalamic lesions, and that ACTH discharge can occur in these rats after injection of large doses of pitressin.

Methods. The method of production of hypothalamic lesions by the Krieg instrument and the analysis for adrenal ascorbic acid concentration have been described previously(3). Rats were tested 3 or more weeks after lesions had been placed. Unilateral adrenalectomy under ether anesthesia has been used as the stimulus in all experiments, the ascorbic acid depletion produced in the second gland one hour after removal of the first adrenal serving as the criterion for ACTH secretion. In a number of experiments drugs were injected immediately after removal of the first adrenal to assess their effect on pituitary ACTH discharge. ACTH was injected intraperitoneally in a dose of 3 I.U.†/100 g of body wt., *i.e.*, 10 I. U./animal. Intravenous injection of epinephrine, 5 micrograms/rat (made up in saline at pH 2), of histamine acid phosphate, 3 mg/rat, of pitocin, 1 U./rat, or of varying doses of pitressin (Parke-Davis) up to a maximum of 5 U./rat, was carried out over a 5 minute interval. In additional experiments rats with lesions were injected subcutaneously for 6-14 days with pitressin tannate in oil, in a single daily dose (0.1-0.8 U./day) sufficient to control diabetes insipidus. Daily water intake was measured on several occasions in all rats with lesions; measurements reported were carried out 10 or more days after production of lesions. Hypophysectomized rats obtained from Hormone Assay Laboratory (Chicago) were injected with 5 U. pitressin as described above 3-4 days post-hypophysectomy, and the adrenal ascorbic acid depletion was determined. The hypothalamus and pituitary were serially sectioned at 15-20 micra and stained

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† One International Unit of ACTH is the activity of 1 mg of the International Standard.

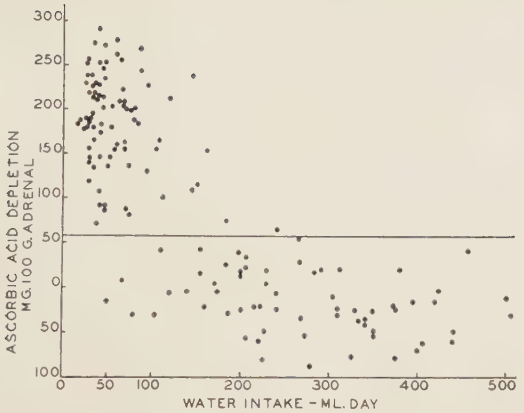

FIG. 1. Relation between polydypsia and adrenal ascorbic acid depletion induced by stress. Daily water intake represented on abscissa; values in excess of 49 ml are indicative of diabetes insipidus. Adrenal ascorbic acid depletion produced one hr after stress of unilateral adrenalectomy is represented on ordinate; values in excess of 58 mg/100 g adrenal (horizontal line) are significantly different from zero. Rats without diabetes insipidus always gave ascorbic acid depletion. Rats with mild diabetes insipidus gave a variable response, whereas rats with water intake in excess of 200 ml/day uniformly failed to respond with adrenal ascorbic acid depletion.

with thionin in 92 and 30 cases, respectively.

Results. Relation between polydypsia and the adrenal ascorbic acid depletion from operative stress. The adrenal ascorbic acid depletion observed one hour after removal of the first adrenal gland of rats with lesions is compared with their daily water consumption in Fig. 1. It can be seen that rats with a water intake within the normal range always manifested ascorbic acid depletion. In rats with mild diabetes insipidus the results were variable; most rats gave ascorbic acid depletion, but occasional rats failed to do so. The number of rats exhibiting ascorbic acid depletion progressively diminished as the diabetes insipidus became more pronounced. Rats with a water intake in excess of 200 ml/day did not develop significant ascorbic acid depletion in any of the 56 cases tested.

In Fig. 2, the mean adrenal ascorbic acid depletion for each increment in water intake is plotted against the logarithm of water intake. Ascorbic acid depletion remains constant at approximately 190-200 mg % until a water intake of about 60 ml per day is reached. From this point on the depletion becomes progressively less, and the relationship is linear until no depletion is produced beyond 195 ml water intake per day. The regression calculated for the linear portion of the curve adequately describes the data presented in Fig. 1 in the range between 40 and 220 ml water intake.

Effect of various agents on the adrenal ascorbic acid in rats with severe diabetes insipidus. In subsequent experiments, rats with a water intake of 200 ml/day or more were used as assay animals to evaluate the effect of various agents (Table I). Since unilateral adrenalectomy *per se* did not deplete the ascorbic acid of such rats, any ascorbic acid depletion observed presumably would be due to the drug administered. ACTH produced a definite ascorbic acid depletion in all 5 rats tested, the mean response being 158 mg %. Intravenous epinephrine failed to induce significant ascorbic acid depletion in 4 rats. Histamine produced a significant depletion of ascorbic acid in one of the 6 rats tested; the mean depletion of 30 ± 17 mg % was not significant. Intravenous pitocin was also without effect. Administration of pitressin tannate in dosage sufficient to return the water intake of 6 rats with severe diabetes insipidus

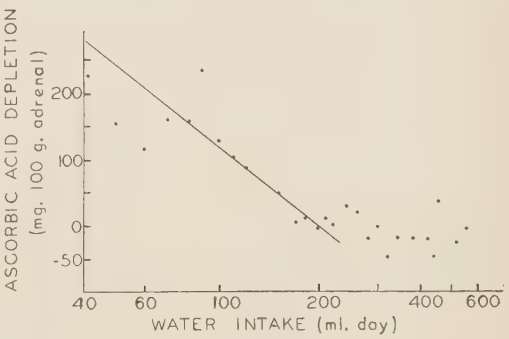

FIG. 2. Relation between logarithm of water intake and adrenal ascorbic acid depletion. Logarithm of water intake is represented on abscissa; adrenal ascorbic acid depletion on ordinate. Each point represents mean adrenal ascorbic acid depletion of all rats for each 10 ml increment in water intake. There is a linear relation between logarithm of water intake and ascorbic acid depletion in the range from 60 to 200 ml water intake/day. Regression calculated for this range of water intake is $y = 923 - 403 \log W$, where y is ascorbic acid depletion in mg/100 g adrenal and W is water intake in ml/day. Standard deviation about regression line is 39, and standard error of slope (Sb) is 72.

TABLE I. Ascorbic Acid Depletion Produced by Ether and Unilateral Adrenalectomy as Influenced by Various Procedures.

Procedure	No. of rats	Adrenal ascorbic acid depletion (mg/100 g adrenal) Individual response	Mean ± S.E.*	Daily water intake (ml/24 hr)
A. Rats with severe diabetes insipidus:				
1. Unilateral adrenalectomy, untreated	56	——	−14 ± 5	307
2. Unilateral adrenalectomy plus:				
a. ACTH, 10 mg I.P.†	5	154, 183, 235, 92, 124	158 ± 28	284
b. Epinephrine, 5 γ I.V.†	4	28, 3, −12, −16	1 ± 10	259
c. Histamine acid PO_4, 3 mg I.V.	6	−2, 24, 59, −3, 99, 6	30 ± 17	297
d. Pitocin, 1 U., I.V.	4	−17, 30, 0, 22	2 ± 12	254
e. Pitressin in oil†	6	15, −23, 33, −4, 28, −5	7 ± 9	47
f. Aqueous pitressin,				
1) 5 U., I.V.	6	91, 50, 244, 179, 177, 178	150 ± 28	274
2) 2.5 "	3	89, 216, 25	110	291
3) 1.25 "	3	63, 52, 42	52	300
4) .61 "	4	79, −16, 39, 34	34 ± 19	384
B. Hypophysectomized rats:				
Aqueous pitressin 5 U., I.V., immediately after unilateral adrenalectomy	6	−24, 35, −26, 39, 14, −15	4 ± 12	

* Stand. error of mean.

† I.P. = intraperitoneally; I.V. = intravenously. All treatment was given immediately after removal of the first adrenal and 60 min. before the second was taken for ascorbic acid depletion. In the case of pitressin tannate in oil, the hormone was given subcutaneously to control diabetes insipidus for 6-14 days before testing by unilateral adrenalectomy.

to normal or near normal levels for 6-14 days did not alter the expected results of unilateral adrenalectomy in these rats. There was no significant restoration of ascorbic acid depletion. The pre-pitressin water intake of these 6 rats averaged 408 ml/day. During the last 2 days on pitressin therapy this was reduced to an average of only 47 ml/day.

As a final experiment in this series the effect of intravenous pitressin was evaluated. Five U. of pitressin were administered intravenously to 6 rats with lesions and severe diabetes insipidus and to 6 recently hypophysectomized rats. The only adverse reaction from this injection consisted of slight respiratory depression in several rats and a perceptible blanching of the skin. Ascorbic acid depletion now developed in rats with lesions although there was considerable variability. No ascorbic acid depletion resulted in the hypophysectomized rats. The response to pitressin in rats with lesions decreased along a log dose response curve. The minimal effective dose of pitressin in rats with lesions appeared to be > 0.6 U./rat.

Relation between polydypsia and adrenal weight. The adrenal weight of rats with lesions is compared with their daily water intake in Fig. 3. There was a reduction in adrenal weight of rats with diabetes insipidus which was directly proportional to the water intake up to a level of 150 ml per day. At this water intake the adrenal weight was roughly half that found in rats without diabetes insipidus or in unoperated controls. At the 150 ml level there was an inflexion in the curve, and from this minimum value adrenal weight increased directly with water intake until supernormal values were reached at water intakes in excess of 350 ml per day. The adrenal weight changes found in rats in which the lesions directly involved the portal system were similar to those found in rats in which this system sustained little or no direct damage. It is, therefore, reasonable to assume that these weight changes did not depend on variations in adenohypophyseal blood supply.

Control of the severe diabetes insipidus for a period of time with pitressin in the 6 rats previously mentioned reduced the adrenal weight of these animals to 37 ± 4 mg, a value which was significantly different ($P = 0.05$) from the mean of 45 ± 2 mg found in 21 rats with similar water intakes which were not so

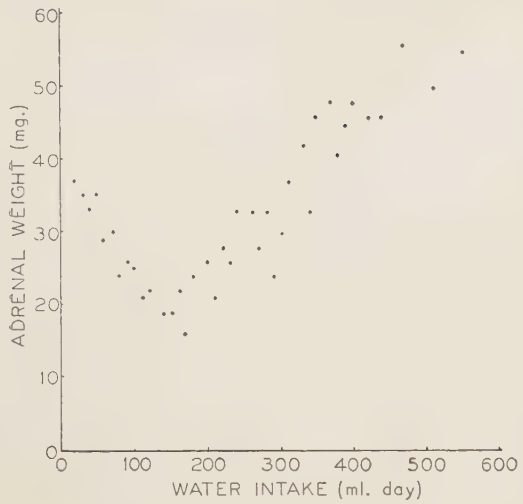

FIG. 3. Relation between polydypsia and adrenal wt. Water intake is plotted on abscissa; adrenal wt on ordinate. Each point represents mean adrenal wt of all rats for each 10 ml increment in water intake. If it is assumed that the curve is composed of 2 straight lines of opposite slope intersecting at a value for water intake of approximately 150 ml/day, 2 regression lines can be used to describe the data. Their equations are respectively $y = 39 - 14.8x$ and $y = 4.5 + 9.9x$, where y = adrenal wt in mg and x = water intake in 100 ml/day; standard deviations about the regression lines are 1.7 and 4.2, and standard errors of the slopes (Sb) are 1.19 and .76, respectively.

treated. When adrenal weight was plotted against water intake as in Fig. 3, the correlation coefficient was $r = 0.94$. This value was significantly greater ($P < 0.02$) than the value obtained ($r = 0.268$) by plotting the adrenal weight of the pitressin treated rats against their pre-pitressin water intake.

Comparing the relation between adrenal weight and water intake (Fig. 3) with that between ascorbic acid depletion and water intake (Fig. 1 and 2), it was apparent that as the water intake increased both the ascorbic acid depletion from unilateral adrenalectomy and the adrenal weight decreased. The slopes of the 2 regression lines were similar. The water intake at which adrenal weight was minimal was very near the point where rats uniformly failed to develop ascorbic acid depletion. From this point on the 2 curves diverged, and an increase in adrenal weight occurred in spite of the continued absence of ascorbic acid depletion.

The results indicate that destruction of a large percentage of the supraopticohypophyseal tract results in marked adrenal atrophy, but that even under these conditions the stress of severe diabetes insipidus can cause a reversal of this trend with the production of adrenal hypertrophy. This suggests that even with loss of a large percentage of the supraopticohypophyseal tract a small sustained increase in ACTH output can occur in situations of chronic stress although the acute stress response is blocked.

Location of the lesions. Most of the lesions in this series were designed to destroy that portion of the median eminence of the tuber cinereum which lies rostral to the level of separation of the hypophyseal stalk, but in some cases an attempt was made to interrupt the fibers of the supraopticohypophyseal tract before they entered the median eminence. All lesions so far examined which were associated with a blockade of the acute stress response have destroyed a large percentage of the supraopticohypophyseal tract as evidenced by the location of the lesions, decrease in number and/or chromatolysis of the cells of the supraoptic nuclei, atrophy of the ventral wall of the hypophyseal stalk, and atrophy of the infundibular process. Most lesions also involved the paraventriculo-hypophyseal tract or partially destroyed the paraventricular nuclei directly. (The paraventricular nuclei are not essential to ACTH discharge in severe stress since in 3 rats previously studied complete destruction of the paraventricular nuclei failed to prevent the ascorbic acid depletion from operative stress(1)). The tuberohypophyseal tract appears to have escaped injury in 12 rats with effective lesions since the lesions ceased rostral to the separation of the hypophyseal stalk. Apparent destruction of this tract has not altered the stress response in our hands (1). Thus, of the 3 well defined tracts entering the median eminence, only the supraopticohypophyseal tract appears to be causally related to the results.

The median eminence itself has been deliberately injured in most of these rats. However, in 2 rats with blockade of the stress response it appears to have escaped direct injury. In 5 others only the rostral tip was injured, and in 3 others it was intact from the point of

maximal development of the structure to the level of stalk separation. The pars tuberalis and portal vessels appeared to have escaped significant injury in the rats in which the median eminence was largely intact. On the other hand, 3 of the rats responded normally to stress even though the rostral end of the median eminence and portal system were involved. It appears likely, therefore, that destruction of the median eminence is not a necessary condition for blockade of the stress response and that destruction of a large percentage of the supraopticohypophyseal tract is the most constant feature in rats with effective lesions of the hypothalamus.

Discussion. The above results indicate on both anatomical and functional grounds that destruction of the supraopticohypophyseal tract uniformly blocks pituitary ACTH release in response to acute stress. Not only is adrenal ascorbic acid depletion prevented, but the elevation in blood ACTH concentration which normally follows stress also fails to occur in rats with severe diabetes insipidus(4). That injury to the supraopticohypophyseal tract also effects basal ACTH discharge is indicated by the reduction in adrenal weight which occurs in rats with diabetes insipidus, though this trend is reversed at high levels of water intake. The results obtained on plotting adrenal ascorbic acid depletion, adrenal weight, and blood ACTH concentration against water intake are given in Fig. 4. It is apparent that the slopes of all 3 regression lines are similar.

The blockade of the acute stress response is not due to the severe disturbance in water balance which ensues since restoration of water intake to normal levels does not restore the response. The adrenals of rats with effective hypothalamic lesions still retain the ability to respond to exogenous ACTH administration with ascorbic acid depletion. They do not respond to operation, exogenously administered histamine, epinephrine or pitocin. If large doses of pitressin are administered ascorbic acid depletion develops which is probably not due to ACTH contamination of the extract since ascorbic acid depletion does not occur in the hypophysectomized rat. The results indicate that large doses of pitressin as used

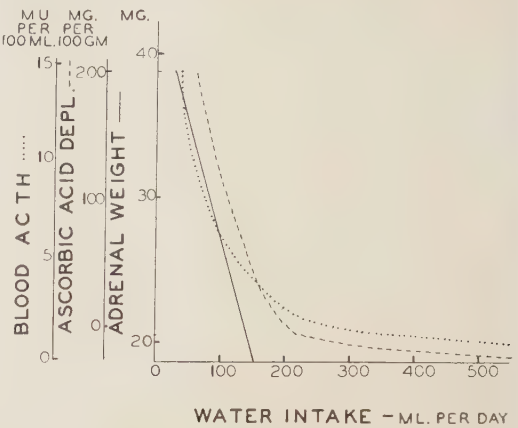

FIG. 4. Relationship between 3 indices of ACTH secretion and water intake in rats with hypothalamic lesions. Water intake is represented on abscissa; values for adrenal ascorbic acid depletion, adrenal wt, and blood ACTH concentration on ordinate. Solid, dashed, and dotted lines represent regressions calculated for adrenal wt, adrenal ascorbic acid depletion, and blood ACTH concentration, respectively. Values for blood ACTH concentration are calculated from data of McCann and Sydnor(4).

here can activate the anterior lobe to discharge endogenous ACTH. This appears reasonable since a significant store of hypophyseal ACTH still exists in rats with hypothalamic lesions (4). Since other stimuli such as operation, histamine, and epinephrine injection fail to discharge ACTH in these preparations, it appears likely that the action of the pitressin was directly on the hypophysis either by way of an effect on its vasculature or on the parenchymal cells. These experiments do not determine with certainty the nature of the active material, but, in all probability, it was either the pressor factor or antidiuretic hormone since pitocin at a dosage level 4 times that which existed in the largest dose of pitressin failed to produce ACTH secretion.

The dosage of pitressin employed to induce ascorbic acid depletion in rats with lesions was large, and the minimal effective dose of approximately 0.6 U. is greater than that required to produce antidiuresis(9a). Several possibilities may explain this relative insensitivity to pitressin. In the first place, the half life of intravenously injected pitressin is approximately 50 seconds(10); this means that an effective concentration probably reached

the hypophysis for only a short interval in these experiments. The concentration of antidiuretic hormone (pitressin) which normally reaches the anterior lobe may be much higher than that in the systemic circulation, since the anterior lobe receives a considerable portion of its blood supply via the hypophyseal portal vessels, and since, in all probability, antidiuretic hormone is secreted directly into the capillaries which coalesce to form the portal vessels in the median eminence(11). Lastly, the sensitivity of the hypophysis and/or the adrenal ascorbic acid test system may well be subnormal in the rat with interruption of the supraopticohypophyseal tract. An analogous situation is the reduced sensitivity of the adrenal to ACTH in rats tested long after hypophysectomy(12).

The evidence for the importance of other postulated mechanisms regulating ACTH secretion which these lesions might block is not convincing. The fact that ACTH discharge still occurs after neurological stalk section clearly rules out a direct secretomotor innervation of the anterior lobe as a necessary factor in ACTH secretion(5,6,7). The presence of histologically normal anterior lobes in rats and cats with effective lesions argues against the hypothesis that the results are due to inadequate blood supply to the adenohypophysis(1,3,4), as does the observation that the hypophyseal portal system appears to be intact in some of these rats. Rats with a considerable adrenal atrophy may still manifest ascorbic acid depletion, while this response may be absent in animals showing a marked adrenal hypertrophy. According to the hypothesis of impaired hypophyseal blood supply, one would expect to find a positive correlation between adrenal size and ACTH release.

The blockade in ACTH release associated with destruction of the supraopticohypophyseal system together with the ACTH secretion produced by one of the hormones known to be secreted by this system strongly suggests that the supraopticohypophyseal system in large measure controls pituitary ACTH output by release of antidiuretic hormone directly into the portal circulation during stress. Considerable additional evidence is in harmony with this view. Antidiuretic material can be extracted from the hypothalamus(13). Antidiuretic hormone is almost certainly released into the circulation during stress; this was first clearly shown by the experiments of Verney and his colleagues on the antidiuresis associated with emotional stress(14). Rothballer has shown that the "neurosecretory" material found in the neurons of the hypothalamohypophyseal system is depleted from the median eminence and infundibular process after painful stimuli(11). Mirsky, *et al.* have reported an acute elevation in blood antidiuretic hormone titer after various stimuli, such as pain and adrenalectomy(15), all associated with augmented ACTH discharge. The time relations of the rise in antidiuretic hormone concentration parallel the increase in blood ACTH after stress (15,16,17). This correlation between increased antidiuretic hormone release and increased ACTH discharge has already prompted Rothballer(11) and Mirsky(15) to consider antidiuretic hormone as a possible neurohumoral transmitter agent.

Conclusions. 1. Hypothalamic lesions which block ACTH secretion, as judged by adrenal ascorbic acid depletion, adrenal weight, or blood ACTH concentration, uniformly destroy a significant fraction of the supraopticohypophyseal tract as evidenced by their location and the presence of diabetes insipidus. 2. ACTH secretion appears to be produced in these rats by large doses of pitressin. 3. Control of diabetes insipidus by small doses of pitressin, or the injection of epinephrine, histamine or pitocin does not produce significant ACTH secretion in such rats. 4. The results indicate that the supraopticohypophyseal tract may play a role in the regulation of ACTH secretion by release of antidiuretic hormone into the hypophyseal portal vessels.

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Effects of Hormone Administration on Serum Protein Patterns.* (21369)

CORNELIA HOCH-LIGETI AND KAREN IRVINE.
From the Department of Pathology, School of Medicine, University of Virginia, Charlottesville.

In a previous paper(1) changes in the serum protein patterns of patients undergoing surgical operation, as found by the use of paper electrophoresis, were described. These consisted of a significant decrease in the albumin concentration and an increase in the concentration in the alpha$_1$ and alpha$_2$ globulins during the first 4 days following operation. Thereafter the serum components tended to return to their preoperative levels. The changes in the serum protein patterns were discussed in relation to the protein disequilibrium which occurs after surgical or accidental trauma(2,3). Injuries are followed by a negative nitrogen balance with increased nitrogen excretion, which in turn is succeeded by nitrogen retention, probably because of increased tissue anabolism. The mechanism and biological significance of the negative nitrogen balance after injury is not clear. It is generally assumed that the direct destruction of tissues during operation is not the responsible factor. There are many similarities in the nitrogen metabolism in response to injury and the general "alarm" or "adaptation syndrome" described by Selye(4). The administration of the adrenocorticotropic hormone of the pituitary and of adrenal cortical steroids, such as 11-oxy corticoid, is reported to produce a negative nitrogen balance(5,6).

In order to find out whether the changes in the nitrogen balance induced by various hormones are associated with changes in the serum proteins, the effect of hormone injections on the serum proteins of rats was studied. The effect of hormones on the maintenance of the serum protein level in experimental animals has been investigated previously, both by the methods of administration of hormones and by removal of endocrine glands(7-9). The findings of these experiments suggest that the hormones of the pituitary, adrenals and possibly thyroid are the main hormonal regulators of the composition of serum proteins. It was thought that adrenal steroids affect the gamma globulin levels by releasing this protein from the lymphocytes(10), but these findings could not be substantiated(11).

Materials and methods. Most experiments were performed with young female Wistar rats, males also being used when a difference in response to hormones due to sex could be expected. The rats were fed a full diet, food and water was given *ad libit.* Sham operation consisted of opening the abdomen, manipulating the intestines gently with an instrument, and closing the abdomen by sutures. In op-

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TABLE I. Effect of Surgical Operation and Hormone Administration on Serum Protein Patterns of Rats.

No. of rats	Treatment	Simple dose, mg	Total protein, g/100 ml	% of total protein: Albumin*	Alpha$_1$*	Alpha$_2$*	Beta*	Gamma*
9	Control, A†	—	6.5 ± .34	46.4 ± 3.9	10.2 ± 1.8	12.8 ± 1.9	17.1 ± 1.9	12.6 ± 1.7
7	" , B†	—	6.7 ± .36	43.4 ± 1.6	10.4 ± 1.8	15.4 ± 2.1	17.0 ± .9	13.5 ± 2.0
9	Operation	—	6.4 ± .53	36.9 ± 2.7	10.3 ± 2.3	15.7 ± .8	20.8 ± 1.8	13.5 ± 1.7
4	Narcosis only	—	—	43.4 ± 3.8	9.7 ± 2.7	12.3 ± 4.3	18.9 ± 5.9	13.6 ± 4.0
10	ACTH‡	2 u.	7.0 ± .54	39.2 ± 4.5	14.1 ± 2.5	16.5 ± 2.3	18.2 ± 2.0	12.8 ± 2.0
5	Thyrotropin	5.0	7.0 ± .36	41.5 ± 4.8	12.1 ± 5.8	13.4 ± 4.3	18.5 ± 1.9	13.9 ± 5.3
5	Growth hormone	3.0	6.3 ± .8	45.0 ± 2.7	10.9 ± 1.2	13.9 ± 1.6	16.3 ± 2.8	10.0 ± 8.7
5	F.S.H.‡	1.0	7.2 ± .37	42.5 ± 8.0	13.0 ± 2.5	14.2 ± 3.1	15.9 ± 1.1	15.9 ± 3.5
5	Cortisone	12.5	7.7 ±6.4	51.2 ± 3.7	9.5 ± 0.7	11.9 ± 0.7	17.4 ± 1.9	10.2 ± 4.1
4	"	6.25	—	49.2 ± 7.1	10.7 ± 2.5	13.7 ± 2.0	17.3 ± 3.5	11.8 ± 5.3
10	Hydrocortone	5.0	7.0 ± .35	52.1 ± 3.0	13.3 ± 1.4	10.2 ± 1.3	16.2 ± 1.5	7.9 ± 1.8
5	D.O.C.A.‡	.5	—	44.5 ± 2.4	9.3 ± 1.8	13.4 ± 4.3	17.5 ± 1.2	13.1 ± 1.3
5	Cortical ext.	1.25 u.	7.0 ±1.1	46.2 ± 6.1	14.1 ± 2.5	11.4 ± 2.1	17.3 ± 2.3	13.6 ± 3.9
4	Nor-epinephrine	.02	—	45.7 ± 7.8	11.4 ± 1.5	12.3 ± 3.0	17.0 ± 3.9	12.3 ± 2.3
5	Estrone	.02	—	44.4 ± 2.5	10.1 ± 2.9	15.4 ± 5.8	17.4 ± 3.3	11.7 ± 2.0
5	Progesterone	.25	6.8 ± .82	46.6 ± 3.8	12.7 ± 1.7	11.7 ± 1.9	17.2 ± 1.9	13.1 ± 2.7
5	Testosterone ♀	10.0	6.8 ± .84	54.3 ± 3.8	9.3 ± .7	11.5 ± 2.0	15.7 ± 3.2	9.3 ± 2.8
5	" ♂	10.0	6.0 ± .41	47.5 ±12.7	15.5 ± 8.6	12.8 ± 6.5	15.0 ± 5.6	9.7 ± 3.0
9	Insulin	.5 u.	7.0 ± .78	43.6 ± 2.6	14.8 ± 1.2	12.2 ± 1.3	17.5 ± 2.0	14.2 ± 1.3
5	Thyroxine	.04	6.3 ± .71	44.5 ± 2.0	10.9 ± .9	13.6 ± 2.1	17.3 ± 1.2	14.2 ± 2.7

* Data are median values and 95% confidence intervals for each group. Significant findings underlined.

† Control A = Untreated rats killed by ether. Control B = Untreated rats killed by breaking the neck.

‡ ACTH = Adrenocorticotropic hormone. F.S.H. = Follicle stimulating hormone. D.O.C.A. =Desoxycorticosterone acetate.

u. = units.

erated groups rats were killed on successive days following operation. Groups of 5 rats were employed for screening of a hormone. These rats received an injection on one day; every following day one rat was killed and the survivors again injected. Accordingly, the last rat killed had received 4 times the initial dose. In several instances the experiment was repeated with another group of 5 rats and the results pooled. Blood was drawn from the heart of each rat, after which it was killed, the body, adrenal and ovarian weights were recorded, and the organs fixed for histological investigation. It was found that the serum protein patterns did not differ whether the rat was killed by narcosis or by breaking the neck. As blood is drawn more easily from the rats killed by ether narcosis, the rats used for the screening of hormones were killed in that manner. Findings on rats showing any disease at autopsy were discarded. The total serum protein was determined by a modified Linderström-Lang specific gravity gradient (12). The serum protein patterns were studied by the use of the paper strip electrophoresis as described by Durrum(13); the concentrations of the components were expressed as percentage of total protein. When an alteration in the serum composition occurred, it was generally manifest after the first injection. The degree in the change of the serum component and the amount of hormone administered was, however, not in linear relation. This might be partly due to variations in the individual starting levels, which were not determined. Values of serum components found in every set of experiments are compared to the values for untreated rats. The significance of the findings was evaluated by the use of the simplified statistics for small numbers of observations(14).

Results. In Table I the effects of operation and of the administration of hormones on the serum protein patterns are summarized. The total protein concentration of the sera of rats did not change significantly under the influ-

TABLE II.
Body and Organ Weights of Rats following Operation or Administration of Hormones.

No. of rats	Treatment	Single dose, mg	Body wt, g	Adrenal, % body wt	Ovary, % body wt	Ovary, mg
9	Control A	—	107 ± 18.5	27.4 ± 2.7	67.6 ± 14.2	95.2 ± 23.8
7	" B	—	110 ± 27.0	27.0 ± 6.1	—	—
9	Operation	—	130 ± 27.0	35.5 ± 6.5	61.3 ± 11.5	80.3 ± 20.1
5	Narcosis only	—	128 ± 15.8	31.8 ± 7.7	—	—
10	ACTH	2 u.*	160 ± 46.4	35.7 ± 2.4	48.0 ± 11.8	90.0 ± 33.7
5	Thyrotropin	5.0	213 ± 25.5	31.9 ± 6.2	61.5 ± 21.6	131.3 ± 58.0
5	Growth hormone	3.0	93 ± 20.9	30.0 ±13.3	52.3 ± 21.2	46.3 ± 15.8
5	F.S.H.	1.0	190 ± 15.3	32.2 ± 2.4	71.3 ± 24.0	122.1 ± 29.0
5	Cortisone	12.5	106 ± 36.7	22.7 ± 2.8	—	—
10	Hydrocortone	15.0	97 ± 12.5	21.9 ± 2.9	58.1 ± 18.1	52.7 ± 15.6
5	D.O.C.A.	.5	115 ± 15.3	23.6 ± 2.0	—	—
5	Cortical ext.	1.25 u.	139 ± 48.5	35.7 ± 2.4	63.8 ± 13.8	100 ± 17.0
5	Estrone	.02	120 ± 18.8	29.5 ± 5.0	—	—
5	Progesterone	.25	125 ± 18.8	30.7 ± 4.6	44.1 ± 13.1	49.3 ± 20.4
5	Testosterone ♀	10.0	190 ± 20.8	30.8 ± 2.0	44.4 ± 21.0	79.5 ± 36.2
5	" ♂	10.0	198 ± 16.4	18.8 ± 5.8	—	—
9	Insulin	.5 u.	190 ± 25.0	29.0 ± 5.3	46.0 ± 5.3	101.0 ± 2.6
5	Thyroxine	.04	139 ± 2.6	20.4 ± 2.8	59.1 ± 9.3	82.5 ± 12.6

Data are median values and 95% confidence interval for each group. Significant findings underlined.

* u. = units.

ence of operation. Similar to the findings on human patients after operation, the albumin concentration decreased in sera of operated rats. In human sera the $alpha_1$ and $alpha_2$ globulins were increased; in the sera of rats the beta globulin was found to be increased after operation. The serum proteins of rats returned to normal after about 4 days. Narcosis alone without opening the abdomen had no significant effect on the serum protein patterns.

The administration of hormones did not affect the total serum proteins with the exception of large doses (12.5 mg) of cortisone, which increased the serum protein concentration. Of the pituitary hormones investigated, adrenocorticotropic hormone (ACTH) was found to produce a decrease in the serum albumin similar to that occurring after operation. The decrease was, however, less marked. Thyrotropic hormone from beef pituitary slightly decreased the albumin concentration. Pituitary growth and follicle stimulating hormones had no effect on the serum protein patterns in the doses employed.

Of the adrenal steroids, cortisone had no effect on the serum albumin when injected at a level of 6 mg and produced a slight increase at a higher level. Hydrocortone effected a significant decrease in the gamma globulin concentration and increased the albumin concentration. Desoxycorticosterone and adrenal cortical extract (Upjohn) were without effect. Norepinephrine, the hormone of the adrenal medulla, produced no alteration in the serum protein patterns.

Estrogen and progesterone were without effect on the serum albumin. In 2 out of 5 rats estrogen provoked an increase in the $alpha_2$ globulin, which appeared as a double band. Injection of testosterone raised the concentration of serum albumins in female rats. In the male rats the effect was not uniform; out of 5 rats, only 3 showed an increase of the serum albumins. Insulin administration increased the $alpha_1$ concentration.

Table II shows the variation of the body, adrenal and ovarial weights of the rats. The adrenal weight is given as percentage of body weight, and the weight of the ovaries is given both as mg weight and as percentage of body weight. The weight of the adrenals increased following operation and under the influence of ACTH and thyrotropin. The possibility

that the effect of thyrotropin might be due to the presence of ACTH in this preparation should be considered. The increased weight of the adrenal on administration of cortical extract lacks an explanation at present. The weights of adrenals were decreased when cortisone, hydrocortone, or desoxycorticosterone were injected. The weights of adrenals of male rats, injected with testosterone, were very low (18.8 ± 5.8); however, the value was not significantly different from adrenal weights of untreated male rats (20.6 ± 4.2).

The evaluation of changes of ovarial weights is rendered difficult because of the large variations in the weight of these organs. Significant increase in the mg weight was found after injection of follicle stimulating hormone, and significant decrease after injection of progesterone and testosterone.

Routine hematoxylin-eosin stain failed to reveal any constant change in the endocrine organs of the rats except in those treated with thyrotropic hormone, where an increase in the colloid in the follicle and an infolding of the follicle epithelium was prominent after the hormone injections.

Discussion. This series of experiments represents a screening test of hormonal effects on serum protein patterns. It is of interest that although a certain correlation between the effect of hormones on the nitrogen equilibrium and on the serum protein patterns could be found, this correlation is by no means complete. ACTH, which produces a negative nitrogen balance, decreases the serum albumin content. Testosterone, which produces a positive nitrogen balance, increases the serum albumin concentration, particularly in females. The estrogenic hormones do not affect the nitrogen equilibrium and are also without effect on the serum protein patterns. It is, however, puzzling that all adreno-cortical hormones tested, including cortisone, were without effect on the serum albumin concentration. In experiments on nitrogen balance, both ACTH and cortisone produced a negative nitrogen balance(5). White and Dougherty (15), however, found that the injection of adrenocorticotropic hormone or adreno-cortical steroid during the first 24 hours increases the total serum proteins of rats, after which time the level is somewhat lower. In a review of the protein metabolism in disease by Pollack and Halpern(2), the conclusion is reached that the presence of adrenal cortex is essential to the metabolic response to injury or disease, but that adrenal cortical activity may not by itself be responsible for the altered protein metabolism. In the experiments presented, pituitary adrenocorticotropic hormone produced changes in the serum protein patterns similar to those after trauma, but these changes are not mitigated by any of the adrenal cortical hormones tested. It should be remembered that cortisone causes a water shift in the body, and the increase of the albumin and total protein concentration might be due to that phenomenon.

The decrease of gamma globulins in serum after injection of hydrocortone might be connected with the depression of the body response to injury by this hormone, but since the effect on gamma globulins is not shared with cortisone, its biological significance is not obvious.

With the exception of the increase in the adrenal weight on administration of cortical extract (Upjohn), the changes in the weights of the adrenals and ovaries were in exact agreement with the effects expected of the specific hormones.

Summary. 1. In continuation of investigations on changes of serum protein patterns following operation, the effect of administration of hormones on the serum proteins of rats was tested. 2. The total serum protein concentration increased on cortisone administration; no other hormone tested had a significant effect. 3. Pituitary adrenocorticotropic hormone decreased the serum albumin concentration. Testosterone, hydrocortone, and cortisone increased the serum albumin concentration. 4. Insulin increased the concentration of $alpha_1$ globulins. Estrogen produced an increase in the $alpha_2$ globulin concentration, which appeared as a double band. 5. Hydrocortone, but not cortisone, depressed the serum gamma globulin concentration. 6. The other pituitary and adrenal hormones, progesterone and thy-

roxine, were without effect on the serum protein pattern.

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Aedes nigromaculis (Ludlow), Mosquito Naturally Infected with Western Equine Encephalomyelitis Virus. (21370)

J. S. BLACKMORE AND J. F. WINN. (Introduced by M. Schaeffer.)

From Communicable Disease Center, Public Health Service, U. S. Department of Health, Education, and Welfare, Montgomery, Ala.

It is well known that mosquitoes must play an important part in the transmission of western equine encephalomyelitis (WEE) virus, and in past studies several species have been found harboring the virus in nature. These include *Culex tarsalis*(1), *Culiseta inornata, Culex pipiens,* and *Anopheles maculipennis freeborni*(2), *Culex stigmatosoma*(3), *Aedes dorsalis*(3,4), *Culiseta melanura* and *Aedes infirmatus*(5). Thus far, no naturally infected *Aedes nigromaculis* have been reported, although Madsen and Knowlton(6) have demonstrated its ability to transmit WEE virus under laboratory conditions. Hammon and Reeves(7), report the transmission of St. Louis virus by this species in the laboratory.

This paper reports the isolation of WEE virus from a pool of 20 *A. nigromaculis* taken by light trap on September 1, 1953 in Weld County, Colo. Twenty other pools of this same species, captured during August and September and comprising 576 individuals, were tested with negative results. This same area during the 1953 summer season furnished numerous other WEE virus isolations from *C. tarsalis* and from bird bloods(8).

Method. The mosquitoes were taken alive and held 24 hours at room temperature to allow digestion of any antibody-containing blood which might be present(9). They then were lightly anesthetized with ether, identified, pooled according to species, and stored at —20°C for a period of 2 to 14 days before testing for virus. To prepare for animal inoculation, each pool was ground in a chilled mortar in 2.0 ml of 20% horse serum-buffered saline, pH 7.4, and centrifuged at 1000 x G for 10 minutes at room temperature. Three-tenths ml of buffered saline containing 1000 units of penicillin and 2.0 mg of streptomycin was added to 1.0 ml of the supernatant material and incubated overnight at 4°C. The

remaining untreated supernatant was ampuled and stored at —70°C for reference purposes in the case of virus-positive pools. The antibiotic-treated supernatant was inoculated in 0.03 ml volume subcutaneously into each of 4 freshly hatched chicks, which were then observed for 72 hours(10). The infected *A. nigromaculis* pool caused weakness and prostration followed by death of all 4 chicks in 30 to 40 hours.

Results. The brains of these chicks were harvested and showed no evidence of bacterial contamination when cultured in thioglycollate medium. Each brain was made into a 10% suspension and passed in additional chicks with deaths occurring as before. Brains of these, as well as the original infected mosquito suspension, were then sent in a frozen state to the Communicable Disease Center, Virus and Rickettsia Section, Montgomery, Ala., where the agent was identified as WEE virus by complement fixation tests and serum neutralization tests in mice. Reinoculation of the original mosquito suspension killed 3 of 4 chicks and 2 of 5 mice.

Although this isolation of WEE virus from a pool of field-caught *A. nigromaculis* poses this species as a potential vector, its role in the virus infection cycle is not yet clear. The number of virus isolations from *C. tarsalis* in the Weld County area(8), strongly indicate that species to be the vector of prime importance. It is possible, however, that *A. nigromaculis* may play a secondary role in the transmission of WEE virus.

Summary. An isolation of western equine encephalitis virus was made from a pool of 20 *Aedes nigromaculis* mosquitoes taken by light trap on September 1, 1953, in Weld County, Colorado. This represents the first isolation of western equine virus from this species. The importance of this mosquito in the virus cycle is unknown.

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Experimental Production of Sarcomas by Methyl Methacrylate Implants.* (21371)

DANIEL M. LASKIN, IRWIN B. ROBINSON, AND JOSEPH P. WEINMANN.
(Introduced by R. J. Winzler.)

From the Department of Oral and Maxillofacial Surgery and Division of Oral Pathology, College of Dentistry, University of Illinois, Chicago.

Oppenheimer, Oppenheimer and Stout(1,2) have shown that many of the high polymers, including polyethylene and polyvinyl chloride film, induce the formation of sarcomas following subcutaneous implantation in rats and mice for periods of one to 2 years. The structural similarity between several of these substances and methyl methacrylate (acrylic resin) suggested the possibility that methyl

* This investigation was carried out under contract with the Medical Research and Development Board, Office of the Surgeon General of the U. S. Army.

methacrylate might also possess carcinogenic properties. Such findings would be significant because this material has been widely used for subcutaneous prostheses and in denture construction.

Material and methods. Methyl methacrylate film approximately 0.008″ thick was prepared from commercially obtained monomer and polymer similar to that used in making dentures. In addition to methyl methacrylate the monomer liquid contained one part per million of hydroquinone as an inhibitor, while the polymer powder contained cadmium oxide pigments. The combined monomer and polymer were compressed into a film and polymerized by heating in a water bath which was brought from 26° to 100°C in one hour, maintained for 3 hours, and then gradually cooled. Pieces of the methyl methacrylate film measuring approximately 1 x 1 cm, with rounded corners, were sterilized in 1:1000 benzalkonium chloride (Zephiran) and embedded subcutaneously in the lateral abdominal wall of 50 six-week-old Harlan strain albino Swiss mice under aseptic conditions. Care was taken to avoid placing the film directly under the midline incision. Centimeter squares of cellophane (Visking 5½ H. S. cellulose sausage casing) were implanted in a second group of 50 mice as a control series since this material had been shown to possess carcinogenic properties in several other strains of mice(1). Failure to produce tumors in these animals might possibly indicate that we were using a tumor resistant strain. The animals were maintained on a routine stock diet of Purina Fox Chow pellets. They were observed daily until the incisions had healed and then weekly to determine the presence of the implants, the degree of host reaction, and the possible formation of tumors. Animals developing tumors were generally sacrificed after 2 to 3 weeks and specimens were taken for histologic examination. The soft tissues adjacent to the implants in the mice which expired without clinical evidence of tumor formation were also studied.

Results. Gross observations. Generally the surgical sites healed uneventfully. Five animals containing methyl methacrylate film and 12 containing cellophane, however, sloughed their implants. Although the slightly thicker methyl methacrylate film was always readily palpated, there was some difficulty during the first few weeks in determining the location of the cellophane implants. As the tissue reaction on the part of the host increased, however, this difficulty was no longer encountered. There appeared to be a somewhat greater host reaction to the cellophane. Although both types of implants seemed to become encapsulated with connective tissue within about 3 weeks, the capsule surrounding the cellophane film felt slightly thicker. After this initial reaction there were no further changes around the implants unless a tumor developed. The first tumor in relation to a methyl methacrylate implant was observed 257 days postoperatively. At that time 20 of the original 50 mice were still living, the remainder having died of causes apparently unrelated to the experimental conditions. Four additional tumors subsequently developed at 405, 438, 454, and 469 days. Based upon the number of animals surviving at the time the first tumor appeared, this gave a 25% incidence of tumor formation.

Only one tumor, occurring 400 days postoperatively, developed in mice containing cellophane implants. Seven of the original 50 mice were alive at that time giving a tumor incidence of 14.3%. One spontaneous tumor, a mammary gland carcinoma, developed in this group.

Generally the tumors were first felt as slight thickenings in the connective tissue capsule immediately surrounding the implants. They grew very rapidly, measuring more than 3 cm in diameter within 2 weeks. As the tumors increased in size they invariably partially or completely enveloped the implants. At autopsy the tumors were found adjacent, but not directly adherent, to the films which could be readily removed from their capsules. No evidence of metastases was found at post-mortem examination.

Histologic Observations. Sections through the area of the implants in the animals which did not develop tumors showed the methyl methacrylate and cellophane films to be encapsulated by connective tissue. The fibrotic reaction was greatest around the cellophane implants. Small foci of inflammatory cells were observed in a few instances adjacent to

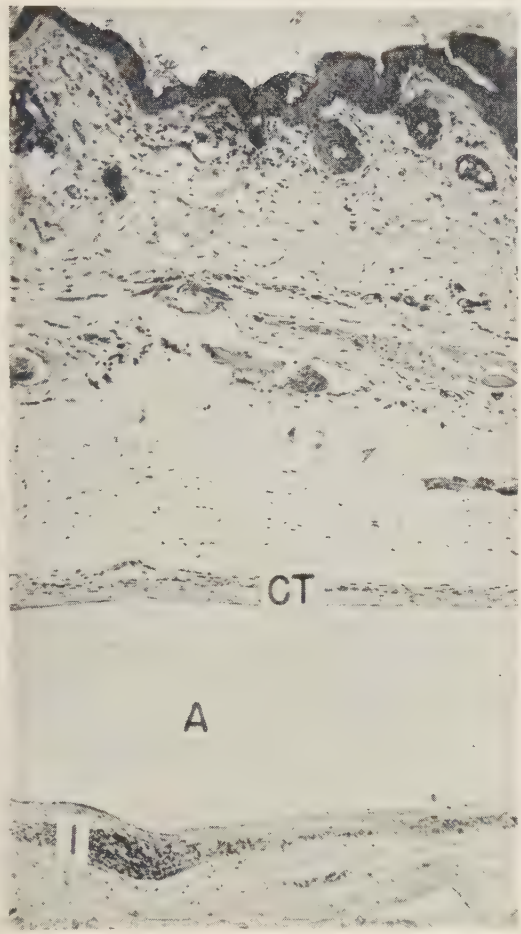

FIG. 1. Section through part of abdominal wall (×77). The space which previously contained the methyl methacrylate implant (A) is surrounded by condensed connective tissue (C.T.). At (I) a chronic inflammatory focus.

both materials (Fig. 1). The tumors which developed from the acrylic and cellophane implants showed considerable structural uniformity and were all diagnosed as fibrosarcomas (Fig. 2).

In one animal in which a piece of cellophane had been embedded, the mammary gland close to the implant developed a tumor diagnosed as an adenocarcinoma. However, a layer of normal glandular tissue intervened between the implant and the carcinoma and for this reason it was classified as a spontaneous tumor.

Discussion. Because of the clinical implications, methyl methacrylate similar to that used commercially in prosthesis construction was selected for these initial experiments. This, however, introduced the possibility that the resulting tumors were due to the presence of either hydroquinone or cadmium oxide. Although it has been suggested that hydroquinone might be carcinogenic(3), it seems unlikely that the minute quantity contained in a centimeter square of methyl methacrylate film would be sufficient to induce tumor formation. Cadmium oxide has been shown to be non-carcinogenic(4). It seems highly probable therefore that the tumors were initiated by the methyl methacrylate itself.

The mechanism of carcinogenesis by high polymeric substances is as yet unknown. Both Fitzhugh(3) and Oppenheimer *et al.*(5) have postulated that the free radical groups formed by these materials might be responsible. Preliminary experiments by the latter investigators, however, seem to indicate that there is no correlation between the carcinogenic effectiveness of the polymers and their free radical content. Alexander(6) has suggested a physical mechanism whereby the films prevent the interchange of metabolites and metabolic products at the site of implantation and thus interfere with normal cell development. In view of the fact that these implants contact only a small area of any given cell, this idea seems rather unlikely.

Methyl methacrylate prostheses have been

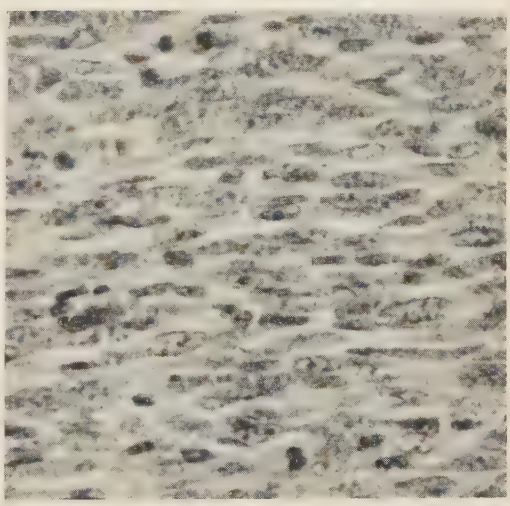

FIG. 2. High magnification (×550) of area from a fibrosarcoma showing predominance of cells, their pleomorphism and large number of mitoses. This tumor developed in relation to a methyl methacrylate implant.

frequently implanted in humans for reconstructive purposes. As yet, no reports of tumor formation have occurred. A latent period of over one year in a mouse, however, might be comparable to more than 20 years in a human subject. Methyl methacrylate was first introduced into clinical use in 1937 and it was not until some time later that it was first used for implantation purposes. It may thus be too early to evaluate the final results.

Summary. Subcutaneous implantation of methyl methacrylate film in mice resulted in a 25% incidence of fibrosarcomas. The incidence of fibrosarcomas was 14.3% with cellophane implants.

Grateful acknowledgement is made to Mr. William Winn for preparation of the photomicrographs.

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Effect of Sodium N-Palmitoyl Sarcosinate* on Tooth Enamel Solubility. (21372)

J. F. VOLKER,† L. S. FOSDICK,‡ R. D. MANAHAN,§ AND R. S. MANLY.‖

Volker reported a simplified method for testing enamel solubility(1). Shortly thereafter he noted that when powdered human enamel was exposed to fluoride solutions it became less soluble in acid. This was the basis for the suggestion that topical applications of sodium fluoride to the enamel surface should be tried as a dental caries preventive measure (2). The majority of clinical investigators who have tested the suggestion by clinical research on children have confirmed the validity of this hypothesis. In the intervening years this method and modifications thereof have been used by several laboratories for the purpose of screening compounds as to their effect on the solubility of enamel in acid. Recently there has been considerable interest in the use of sodium N-lauroyl sarcosinate in the control of dental caries(3). This substance is retained by the tooth surface plaque and has been reported to inhibit acid formation *in situ* (4), although some investigators reported that they were unable to observe such an effect(5).

There is also evidence that closely related homologues have similar prolonged inhibiting effects on acid production in the tooth surface plaque(6). Consequently, it was felt advisable to investigate their possible influence on enamel solubility. Ultimately, these studies led to the observation that sodium N-palmitoyl sarcosinate is markedly effective in reducing enamel solubility.

Methods. The studies were carried out independently in 4 separate laboratories. The methods used in the 4 laboratories were of the same basic type but varied considerably in regard to time of treatment, volumes of solution, etc. Methods are summarized in Table I. The following procedure was used by Volker (1,2) and illustrates the type of test employed. Human enamel powdered to pass a 100 mesh screen was separated and purified by the cen-

* Sodium N-palmitoyl sarcosinate and related homologues were prepared by the Colgate-Palmolive Co. Samples were first submitted to Dr. Manly for testing and his initial findings on reduction of enamel solubility led to extension of the study to the other laboratories.

† University of Alabama, School of Dentistry, Birmingham, Ala.

‡ Northwestern University Dental School, Chemistry Department, Chicago, Ill.

§ Colgate-Palmolive Co., Research and Development Department, Jersey City, N. J.

‖ Tufts College Dental School, Boston, Mass.

TABLE I. Effects of Sodium N-Palmitoyl Sarcosinate and Sodium Fluoride on Enamel Solubility.

Method	Volker: 1 hr treatment of 50 mg 100 mesh enamel with 25 ml of test sol. Sol. decanted, washed 3 times with water, enamel stirred 1 hr in pH 4 acetate buffer. Contents filtered and enamel washed 3 times with water. Wt loss of enamel calculated.		Fosdick: 30 min. treatment of 100 mg 275-300 mesh enamel with 5 ml of test sol. Mixture centrifuged, decanted. Enamel washed 10 times with 10 ml of H_2O for 5 min. Each time centrifuging and decanting. Enamel exposed to 20 ml of pH 4.5 lactate buffer for 30 min. Centrifuged and aliquot of supernatant taken and calcium precipitated as oxalate. Soluble calcium determined by titration.		Manahan: 1 hr treatment of 200 mg of 100-200 mesh enamel with 100 ml of test sol. Mixture filtered, enamel washed 10 times with 50 ml of distilled water. Enamel dried and 50 mg samples added to 20 ml of pH 4 acetate buffer and agitated 1 hr. Mixtures centrifuged, aliquots of supernatant taken and calcium precipitated as oxalate. Calcium determined by titrating with permanganate.		Manly: 3 min. treatment of 50 mg of 60 mesh enamel with 5 ml of test sol. Mixture centrifuged and decanted, enamel is stirred with 6 ml H_2O, centrifuged and decanted. 15 min. stirring in 10 ml of pH 4 acetate buffer. Contents filtered and enamel rinsed with water. Crucible dried to constant wt and wt loss of enamel calculated.	
	Conc. %	Red.	Conc. %	Red.	Conc. %	Red.	Conc. %	Red.
Sodium N-palmitoyl sarcosinate	1	52	.5	52	—	—	1	57,56
	.1	50	—	—	.1	53, 55	.1	52,47,55,35,46
	.01	50	—	—	—	—	—	55, 35, 46
Sodium fluoride	1	48	.5	37	1	44	—	—
	.1	43	—	—	.1	31, 30	.1	34,31
	.01	36	—	—	—	—	—	—

trifugal-flotation method(7). Fifty milligram samples of enamel were placed in previously weighed sintered glass flash funnels (fine porosity) and the funnels, plus samples, were reweighed. To individual 50 mg samples of enamel, 25 cc of distilled water, 25 cc of aqueous sodium fluoride, and 25 cc of aqueous test solutions, respectively, were added. The exit tubes of the funnels were stoppered and the various enamel solutions were agitated with mechanical stirrers for one hour. After treatment, the stopper was removed, the reaction mixtures were pressure filtered and the enamel washed thoroughly 3 times with distilled water. The enamel samples were resuspended in 0.2 M acetic acid/sodium acetate buffer at pH 4. Each suspension was stirred for one hour. The buffer solutions were pressure filtered and the remaining enamel washed three times with distilled water. The funnels with their undissolved enamel were dried in an oven at 100°C for 2 hours and cooled to room temperature in a desiccator prior to weighing. Finally, the funnels were weighed and the weight loss of the original enamel samples was calculated.

Results. Treatment of enamel with 0.1% solutions of sodium N-lauroyl sarcosinate caused a slight but measurable reduction in its solubility in acid (pH 4.0 acetate buffer). Under such conditions, all the investigators obtained an average reduction of less than 5%. One of the investigators (L.S.F.) studied the effect of 0.1% solutions of this compound on the solubility of enamel in acid buffers over the range of pH between 4.0 and 5.4 using the method described under Manahan in Table I. The average reduction in enamel solubility over the entire pH range was 9%. The minimum reduction was 4% at a pH of 4.0 and the maximum reduction was 13% at a pH of 5.0.

All the investigators found sodium N-palmitoyl sarcosinate to be at least as effective as sodium fluoride under comparable test conditions. This relationship held true under a

variety of treatment times, concentrations, etc. The results are summarized in Table I.

In an extension of these observations Volker and Manahan checked the effectiveness of the N-palmitoyl sarcosinate in reducing enamel solubility by measuring the amount of phosphate dissolved by the buffer. Results by this procedure were in good agreement with the gravimetric and calcium titration findings. There was also an indication that foaming must be held to a minimum during the treatment step unless highly purified compound is used. It is also interesting that a 14 hour water rinsing of enamel which had been treated with sodium N-palmitoyl sarcosinate did not result in any appreciable loss of effectiveness.

Studies by one of the investigators (R.D. M.) showed that the sodium N-myristoyl, N-palmitoyl and N-stearoyl sarcosinates were more effective in reducing enamel solubility than the N-lauroyl derivative. A 2% reduction in the solubility of enamel in a pH 4.0 acetate buffer was obtained by treating the enamel for one hour with a 0.1% solution of sodium N-lauroyl sarcosinate. Under comparable test conditions the N-myristoyl, N-palmitoyl and N-stearoyl sarcosinates gave reductions in enamel solubility of 11%, 53%, and 15% respectively. Only the 53% reduction exceeded that obtained with sodium fluoride, which was 31% under these conditions.

Discussion. Since solutions of sodium N-palmitoyl sarcosinate have been shown to be comparable to solutions of sodium fluoride in reducing enamel solubility and since this compound and the closely related sodium N-lauroyl sarcosinate have been reported to have a prolonged inhibiting effect on acid production in the tooth surface plaque, it is suggested that it be further tested in experimental animals to determine if it is effective *in vivo*. Thereafter it should be evaluated in a clinical test in human subjects for the inhibition of dental caries.

Summary. Of the N-acyl derivatives of sodium sarcosine studied, sodium N-lauroyl sarcosinate produced the least effect and sodium N-palmitoyl sarcosinate produced the greatest effect in reducing the acid solubility of powdered human enamel. Aqueous solutions of 1%, 0.5%, 0.1% and 0.01% of sodium N-palmitoyl sarcosinate have been shown to reduce the solubility of powdered human enamel to a degree comparable to that observed with sodium fluoride solutions. These observations may be of importance in the prevention of dental caries.

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Evaluation of Five Methods for Testing Anticonvulsant Activities. (21373)

GRAHAM CHEN, BARBARA BOHNER, AND CHARLES R. ENSOR.

From the Department of Pharmacology, Parke, Davis & Co., Detroit, Mich.

Based upon an antagonism toward electrically- or Metrazol-induced convulsions, the procedures currently used for evaluating anticonvulsant agents are performed under a variety of conditions. The principal variables are: (a) the intensity of a convulsive stimulus, *i.e.* the concentration and route of administration of an analeptic agent, or the frequency and amperage of a current, and (b) the different stages of seizure used as an endpoint for convulsive response. In determining the conditions essential for ascertaining certain spe-

cific anticonvulsant properties of a compound, we have made a comparative study by 5 test procedures of the activities of 10 established central nervous system depressants. The results to be reported in this paper will indicate that the intensity of the convulsive stimulus is of paramount importance in determining the anticonvulsant property of a compound. The nature of the stimulus, electrical or Metrazol, appears to be immaterial. Similar anticonvulsant effects were obtained for the same seizure response with electrically- or Metrazol-induced convulsions. Some quantitative differences in the activities determined by the different methods may be accounted for by the inherent properties of the convulsive stimuli.

Materials and methods. The anticonvulsant agents chosen for our study were: 5,5-diphenylhydantoin sodium (Dilantin sodium); 5 - ethyl - 5 - phenylbarbiturate sodium (phenobarbital sodium); N-methyl-α-phenylsuccinimide (Milontin); 3, 5, 5-trimethyloxazolidine-2, 4-dione (Tridione); N-methyl-α-phenyl-α-methylsuccinimide (PM 396); phenylacetylurea (Phenurone); 5, 5-diethylbarbiturate sodium (barbital); bromodiethylacetylurea (carbromal); 3-o-toloxy-1, 2-propanediol (Mephenesin); and sodium bromide. Tridione, barbital and Mephenesin were given intraperitoneally; the others were administered perorally in a solution or in a suspension of 7% gum acacia. Anticonvulsant activities were determined at the time of their peak effects, the latter having been tested previously. Male mice weighing 18-22 g were used. They were fasted for oral administration 16 hours before use. The test procedures employed were: (a) the supramaximal electroshock seizure technic of Toman, Swinyard and Goodman(1); (b) the "psychomotor" seizure test of Toman(2); (c) the intravenous Metrazol infusion procedure of Orloff, Williams, and Pfeiffer(3), from which a supramaximal chemoshock test (d) was devised; and (e) a subcutaneous Metrazol procedure(4). For supramaximal shock, a 60-cycle alternating current of 24 MA was delivered for 0.2 second to a mouse by clips attached to the two ears. The abolition of the tonic extensor component of the seizure was considered an anticonvulsant effect. For the "psychomotor" seizure test, the parameters of the electrical current were essentially the same as those established by Toman—*viz.*, unidirectional pulses of 1 millisecond duration at a frequency of 6 cycles delivered through corneal electrodes for 3 seconds. For intravenous infusion of Metrazol, a 0.5% solution was administered at the rate of 0.05 cc per 10 seconds; the maximal quantity injected was 1.0 cc per mouse. Two endpoints were taken during injection: (a) the first appearance of clonic seizures and (b) the tonic extension of the hind limbs. The animal was considered to be protected from the extensor component of the seizure when tonic extension of the hind limbs did not occur after 1.0 cc of Metrazol had been delivered. This is approximately 3 times the quantity required to produce a tonic extensor seizure in untreated mice. Usually animals which were protected by an anticonvulsant died without tonic convulsions before 1.0 cc was injected. With subcutaneous Metrazol the animals were given 93 mg/kg of the analeptic agent and observed for convulsive symptoms for 30 minutes. For each test procedure, at least 10 mice were injected with one dose of an anticonvulsant. Three or 4 dose levels, which would protect 10 to 90% of the animals from convulsive seizures, were used to determine a 50% protective dose. The PD_{50} and standard errors were estimated graphically by the method of Miller and Tainter. The standard errors of a ratio of means were computed from the standard errors of the means in percentages (Burn).

Results. In Table I the anticonvulsant activities of the 10 compounds are expressed in terms of the PD_{50} determined by the abolition of the tonic hind leg extensor seizure in electrically- and Metrazol-induced convulsions. The data reveal that a larger protective dose, approximately 2-3 times, was required of each compound for suppressing supramaximal electroshock than for suppressing Metrazol-induced convulsions. It was 6 times in the case of sodium bromide. With the exception of Dilantin, our results are in general agreement with those reported by Goodman and associates(5). They found a smaller dose of Dilantin to protect mice from electroshock

TABLE I. Comparison of Anticonvulsant Activities of Compounds in Mice as Determined by Supramaximal Electro-Shock (S.E.S.) and by Chemo-Shock with a Supramaximal Convulsive Dose of Intravenous Metrazol (S.IV.M.).

(1)	(2)		(3)	(4)	(5)
Compound	Route	Administration Time (hr)*	S.E.S., $PD_{50} \pm$ S.E. (mg/kg)	S.IV.M., $PD_{50} \pm$ S.E. (mg/kg)	S.E.S. / S.IV.M. (range–2 S.E.)
Dilantin	O†	2	9.1 ± .7	4.3 ± .3	2.1 (1.70–2.54)
Phenobarbital	O	2	23.6 ± .8	6.7 ± .9	3.5 (2.54–4.50)
Milontin	O	1	183.0 ± 5.2	92.0 ± 11.2	2.0 (1.49–2.49)
Tridione	IP	1	1320.0 ± 36	345.0 ± 32	3.8 (3.08–4.56)
PM 396	O	1	84.0 ± 3.3	40.5 ± 3.8	2.1 (1.65–2.49)
Phenurone	O	1	110.0 ± 14	39.3 ± 3.4	3.8 (1.94–3.66)
Barbital	IP	2	185.5 ± 8.8	78.0 ± 3.9	2.4 (2.06–2.70)
Carbromal	O	1	150.0 ± 14	52.0 ± 7.3	2.9 (2.11–3.67)
Mephenesin	IP	1/6	147.0 ± 6.4	100.0 ± 9.7	1.5 (1.15–1.79)
NaBr.	O	2	4060.0 ±295	614.0 ± 65	6.6 (4.91–8.31)

* Time after administration. † O = Oral.

than was required to protect animals from maximal Metrazol seizures. This discrepancy in the relative effects of Dilantin is apparently accounted for by differences in the quantities and the rates of intravenous injection of Metrazol employed in the two laboratories. In their tests 38 mg/kg of Metrazol (CD_{96}) was administered in 4 seconds; in ours, one cc of a 0.5% Metrazol solution (250 mg/kg, a supramaximal convulsive dose) was injected at 0.05 cc per 10 seconds.

We are in accord with the views of Goodman *et al.* that, insofar as the tonic hind leg extensor component of the convulsion is concerned, the underlying neurophysiological mechanisms of excitation and recovery appear to be constant and are independent of the initiating stimulus. Identical anticonvulsant activities for a given compound could be obtained against convulsions induced either by a supramaximal intravenous dose of Metrazol or by a current of equivalent intensity. Because a chemical agent is being continuously eliminated at the site of action, Metrazol concentration can never become great enough to produce such high intensity of stimulation as an electrical current can provide. Therefore, the quantitative difference in anticonvulsant activities of the various substances as determined by the supramaximal electroshock and by supramaximal Metrazol seizures is due primarily to a difference in the intensities of stimuli supplied in the two tests.

Before considering the data obtained with the other 3 procedures, it should be pointed out that 2 approaches have commonly been adopted for testing the anticonvulsant activity of a compound by the subcutaneous Metrazol method. In one, the analeptic dose of Metrazol is varied against a fixed dose of an anticonvulsant; in the other, the procedure is reversed with a fixed challenging dose of Metrazol and varying doses of an anticonvulsant. In the former, the anticonvulsant activity of the compound is expressed in terms of a convulsive dose of Metrazol (CD_{50}); in the latter, a protective dose of an anticonvulsant (PD_{50}) is used as an index for anticonvulsant activity. The time-intravenous Metrazol technic of Orloff, Williams and Pfeiffer was devised to re-

TABLE II. Comparison of Anticonvulsant Activities of Compounds in Mice as Determined by "Psychomotor" Test (Ps.S.), Subcutaneous Metrazol (Sc.M.), and by Intravenous Metrazol (a Minimal Convulsive Dose) (M.IV.M.).

(1) Compound*	(2) Ps.S., $PD_{50} \pm$ S.E. (mg/kg)	(3) Sc.M., $PD_{50} \pm$ S.E. (mg/kg)	(4) Ps.S. / Sc.M. (range–2 S.E.)	(5) M.IV.M.,† cc ± S.E. (0.5% Metrazol) μ/20 g	(6) Compound / Control (range–2 S.E.)
Control	—	—		.12 ± .01	
Dilantin	Ineffective at 50 mg/kg	Ineffective at 50 mg/kg	—	.15 ± .01 (50 mg/kg)	1.2 (0.99–1.51)
Phenobarbital	35.5 ± 1.8	31.0 ± 2.0	1.1 (0.9 –1.30)	.28 ± .01	2.3 (1.92–2.75)
Milontin	134.0 ± 6.3	99.0 ± 3.3	1.4 (1.19–1.51)	.19 ± .01	1.6 (1.26–1.90)
Tridione	745.0 ± 58	375.0 ±35	2.0 (1.48–2.44)	.23 ± .01	1.9 (1.56–2.28)
PM 396	104.0 ± 7.4	74.0 ± 3.1	1.4 (1.15–1.63)	.21 ± .02	1.8 (1.32–2.17)
Phenurone	310.0 ± 30.2	126.0 ± 2.9	2.5 (1.98–2.94)	.20 ± .01	1.7 (1.35–1.99)
Barbital	212.0 ± 7.1	82.0 ± 3.1	2.6 (2.36–2.81)	.30 ± .02	2.5 (1.96–3.04)
Carbromal	145.0 ± 12.2	54 ± 7.0	2.7 (1.85–3.53)	.17 ± .01	1.4 (1.14–1.70)
Mephenesin	187.0 ± 6.0	235 ± 7.0	0.8 (0.72–0.88)	.23 ± .03	1.9 (1.32–2.52)
NaBr.	780.0 ± 39	710.0 ± 9.1	1.1 (0.96–1.24)	.24 ± .01	2.0 (1.62–2.38)

* Route of and time after administration were the same as in Table I.

† PD_{50} dose of each compound in column 3 was used to determine amount of 0.5% Metrazol solution required intravenously to produce first clonic movements (a minimal convulsive dose).

place the subcutaneous Metrazol procedure using a fixed dose of an anticonvulsant agent. The subcutaneous Metrazol method developed by us was worked out on the basis of a fixed challenging dose of the convulsant. A comparison of the results obtained by the two avenues of approach is made in the following section.

In Table II are presented anticonvulsant activity data on the ten compounds as obtained by "psychomotor" seizure test, by our subcutaneous Metrazol procedure, and by the time-intravenous infusion of a minimal convulsive dose of Metrazol. In columns 2 and 3, Dilantin is shown to be ineffective at 50 mg/kg in both "psychomotor" seizure and subcutaneous Metrazol test. The PD_{50} values of phenobarbital and sodium bromide are approximately the same determined by either of the two procedures. A larger protective dose, a ratio of 1.5 to 2.5 in PD_{50} values, was required in the "psychomotor" seizure than in the subcutaneous Metrazol method for other compounds (column 4). The relationship is reversed in the case of Mephenesin. This is apparently due to its extremely short-acting property, since a longer period (30 minutes) was used to conduct the subcutaneous Metrazol test than the "psychomotor" seizure test (10 minutes after the administration of Mephenesin). It indicates a limitation in the subcutaneous Metrazol method for determining the peak anticonvulsant activity of an extremely short-acting agent.

It may be remarked here that the stunning reaction observed in electrically-induced seizures is also seen as a premonitory sign in Metrazol-induced convulsions. With a minimal convulsive dose of Metrazol, the "aura" are often the only symptoms seen, especially in drug-treated animals. In our subcutaneous Metrazol test, clonus of the facial muscles and

forelimbs without generalized convulsions is considered to be a positive convulsive response.

In column 5 of Table II are shown the amounts of intravenous Metrazol required to produce the first clonic seizure response in mice to which the anticonvulsants had been administered at PD_{50} doses, determined by the subcutaneous Metrazol test (column 3). The slight difference in the quantities of intravenous Metrazol required for Dilantin-treated and for the untreated mice is not significant statistically. For other anticonvulsants, approximately double the quantity of Metrazol (0.24 cc 0.5%/20 g or 60 mg/kg) was needed for the drug-treated animals as for the untreated controls (0.12 cc 0.5%/20 g or 30 mg/kg). Since the challenging dose of subcutaneous Metrazol, 93 mg/kg, is also approximately twice the CD_{50} (around 50 mg/kg) in untreated mice, and since the PD_{50}s of the anticonvulsants as determined by the subcutaneous test were used for the intravenous Metrazol test, the anti-Metrazol activities of these compounds determined by the intravenous infusion method are practically identical with those obtained by the subcutaneous method.

It may be concluded, then, that the relative anticonvulsant effects of the ten compounds as determined by psychomotor seizure test and by subcutaneous and intravenous Metrazol tests are about the same, Dilantin being ineffective. Because there is a common denominator in the three methods, namely a minimal convulsive response induced by Metrazol or by a current of low intensity, the anticonvulsant effects so ascertained against electrically- or Metrazol-induced seizures are apparently accomplished by the same neurophysiological mechanisms.

Since the primary objective in these tests is to screen compounds for potential antiepileptic activity, a question naturally arises as to which are the most practical methods for such a purpose. Undoubtedly, each test has its own merits under certain conditions. From our experience, the supramaximal electroshock and subcutaneous Metrazol methods are preferred for the following reasons: First, one of the striking features of a drug like Dilantin for grand mal epilepsy is its effectiveness in abolishing the hind leg tonic extensor seizures without exerting any hypnotic and anesthetic action at dosages far above the effective level. By raising the intensity of stimulus above the threshold value, as in supramaximal electroshock, Dilantin-type properties may be distinguished from those of others, like barbital, Mephenesin, and trimethodione, which will inhibit the hind leg extensor seizure response to supramaximal electroshock only at hypnotic or anesthetic dosages(6). Such a distinction is not possible with the intensity of stimuli supplied by intravenous Metrazol. The electroshock procedure is also simpler and less time-consuming.

The procedures employing low intensity of stimulation are used primarily to screen compounds for possible anti-petit mal activity. The "psychomotor" seizure test is a simple procedure; however, the stunning reaction in drug-treated animals is often not distinct. With regard to the two Metrazol tests, the subcutaneous method in our hands is easier and quicker in operation than the time-intravenous infusion method. Furthermore, the anticonvulsant activity of a substance is preferably expressed as in the other tests, in terms of its PD_{50}. This is especially desirable when a comparison of the various anticonvulsant activities of the same substance is required.

Summary. 1. Anticonvulsant activities of 10 CNS depressants were determined by the following 5 procedures: (a) Supramaximal electroshock; (b) supramaximal chemoshock (Metrazol); (c) "psychomotor" seizure; (d) subcutaneous Metrazol; and (e) intravenous Metrazol (a minimal convulsive dose). 2. Results indicate that intensity of convulsive stimuli, electrical or Metrazol, is the important factor in determining anticonvulsant property of a compound. The order of anticonvulsant activities of these compounds is the same whether determined according to the first two methods by the abolition of hind leg extensor seizure, or according to the latter three methods by the inhibition of a minimal clonic seizure response. 3. Reasons are given for our preference of the supramaximal electroshock and the subcutaneous Metrazol method for

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Antigenic Fractions Specific for *Histoplasma capsulatum* in the Complement Fixation Reaction. (21374)

LLOYD J. SORENSEN AND E. EDWARD EVANS. (Introduced by W. J. Nungester.)

From the Department of Bacteriology, University of Michigan, Ann Arbor.

Cross reactions among the species *Histoplasma capsulatum, Blastomyces dermatitidis* and *Coccidioides immitis* have presented considerable difficulty in the use of serological procedures for diagnosis(1-4). In order to secure antigens from *H. capsulatum* possessing greater specificity, fractionation of crude antigens has been initiated(5,6). These studies have employed antigens derived from mycelial histoplasmin(5) or yeast phase cells grown on a solid medium(6). Antigens described in the present study have been isolated from the supernates of fluid cultures of yeast phase *H. capsulatum*.

Methods. Cultures were maintained in pure yeast phase by growth in a neopeptone dialysate medium (NPD)(7,8) under continuous agitation on a rotary action flask shaker at 37°C. *H. capsulatum* strain HC-9 and *B. dermatitidis* B-16 were received from Miss Charlotte Campbell of the Army Medical Service Graduate School, and strain HC-12 of *H. capsulatum* was received from Dr. C. W. Emmons of the National Institutes of Health. For cross-reaction studies, rabbits were infected by the intravenous injection of *C. immitis* spores supplied by Dr. C. E. Smith and additional rabbits were injected with yeast cells of *Sporotrichum schenckii* and *Candida albicans* furnished by Dr. E. R. Harrell. Rabbits were infected with *H. capsulatum, B. dermatitidis* and the other fungi described by injecting 5 x 10^6 cells per kilo of body weight by the intravenous route. Bleedings were made periodically by cardiac puncture, and serum was stored at -20°C. The complement fixation test used was an adaptation of the one described by Bengston(9) and employed by Salvin(2). Adequate controls on hemolytic system, antigen, and antiserum were used routinely. The ground yeast phase antigens of *H. capsulatum* and *B. dermatitidis* were employed in the complement fixation test as standard antigens and were prepared according to the method described by Saslaw and Campbell(10) except that a ball mill was substituted for the Ten Broeck tissue grinder. Whole yeast cell suspensions of *C. albicans* and *S. schenckii*, adjusted to 70% transmission, were used to test the sera from rabbits infected with these fungi. Coccidioidin standardized for use in the complement fixation test was obtained from Dr. C. E. Smith. The purified antigen for *H. capsulatum* was derived from the supernate of yeast phase cell growth in NPD medium. To the supernate was added zinc acetate to a molarity of 0.025 and the pH was adjusted to 7.0 with 10% sodium hydroxide. The precipitate which formed was removed by centrifugation and then dissolved in a quantity of 4M/15 phosphate buffer, pH 7.3 equivalent to 10% of the original volume. The insoluble zinc phos-

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TABLE I. Complement Fixation Titers with Rabbit Serum Withdrawn at 14 and 20 Weeks after Infection.

Antigen	Reciprocal of serum dilution titer — Rabbit No. (*H. capsulatum*)						Rabbit No. (*B. dermatitidis*)					
	H-1	H-2	H-3	H-4	H-5	H-7	B-2	B-4	B-7	B-8	B-9	B-10
318-H9-D	20	80	40	40	20	20	—*	—	—	—	—	—
348-H9-D	80	160		160	40	40	—	—	—	—	—	—
355-H9-D	80	80		40	20	40	—	—	—	—	—	—
Homologous ground YP	40	160	20	40	20	40	160	160	160	160	160	160

Optimal antigen concentration, 0.1 mg/ml with 318-H9-D and 355-H9-D; 0.01 mg/ml with 348-H9-D.

Rabbits H-1, H-2, H-3, H-4, B-2, and B-4 were bled 20 wk after infection; all others were bled at 14 wk after infection.

* Negative at 1:5, the lowest serum dilution used.

phate was removed and after the addition of 10% sodium acetate and one per cent acetic acid repeated chloroform extractions were conducted to remove protein(8). The removal of protein was followed by precipitation with ethanol. The precipitate resulting from the addition of 4 volumes of ethanol was discarded since preliminary tests disclosed that this fraction did not contain the antigen being sought. The addition of a fifth volume of ethanol resulted in a precipitate which was used as an antigen after it had been further purified by reprecipitation, dialysis against distilled water, and lyophilization. Several lots of antigen were prepared with this procedure and, of these, the five used in this study were designated 293-H9-D, 293-H9-G, 318-H9-D, 348-H9-D, and 355-H9-D.

Results. Among the 12 sera listed in Table I, only those from the 6 rabbits infected with *H. capsulatum* fixed complement with the 3 lots of purified antigen. Although serum from the 6 rabbits infected with *B. dermatitidis* yielded titers of 1:160 with the *B. dermatitidis* ground yeast phase antigen and cross reacted with *H. capsulatum* ground yeast phase, they failed to react with the purified antigens. Since the sera used in Table I were taken during the later stages of the disease, it was deemed advisable to test sera from the early period of infection. From Table II it may be seen that the results with sera taken at 2 to 9 weeks post infection were similar to those obtained with the sera taken at 14 to 20 weeks. The 3 purified antigens were both sensitive and specific in tests with sera from rabbits infected with *B. dermatitidis* and *H. capsulatum*. In Table III it will be seen that purified antigen 318-H9-D was also specific when tested against sera from rabbits infected with *C. immitis, S. schenckii,* and *C. albicans*. Each of these sera reacted satisfactorily with the homologous antigen.

Discussion. The antigens prepared in this

TABLE II. Complement Fixation Titers with Rabbit Serum at Different Time Intervals following Infection.

Antigen	Reciprocal of serum dilution titer — *H. capsulatum* infected — HC-3				HC-23				*B. dermatitidis* infected — BD-B				BD-C			
	2*	3	8	9	2	3	8	9	2	3	8	9	2	3	8	9
293-H9-D	40	40	20	10	20	40	20	20	—	—	—	—				
293-H9-G	40	20	40	10	20	40	20	20	—	—	—	—				
318-H9-D					40	160	160	80					—	—	—	—
H. capsulatum																
Ground YP	160	160	40	40	160	80	160	80	40	80	40	40	40	40	20	20
YP supernate	80	80	40	40	40	80	160	80	40	40	40	40	20	20	40	20
B. dermatitidis																
Ground YP									80	160	160	160	160	160	320	160

* Week after infection.

Antigen concentration 0.1 mg/ml.

TABLE III. Complement Fixation Test Using *H. capsulatum* Antigen 318-H9-D and Serums from Rabbits Infected with *C. immitis, S. schenckii, C. albicans, B. dermatitidis,* and *H. capsulatum.*

	Reciprocal of serum dilution titer											
	HC-3			BD-B			CI-1		SS-1		CA-1	CA-2
Antigen	2*	9	20	2	9	20	9	36	2	47	20	20
318-H9-D	80	40	40	—	—	—	—†	—	—	—	—	—
Homologous antigen	160	40	20	80	160	160	800	800	40	40	40	20

* Weeks following infection. † Anti-complementary below 1:40.

study by precipitation with zinc and alcohol may provide an answer to some of the problems in serodiagnosis of histoplasmosis and related infections. With serum from infected rabbits, these antigens have exhibited good sensitivity and high specificity. The question of whether they will behave in a similar manner with serum from infected humans remains to be answered. The antigens described in this report appear to differ from those reported by Campbell(6) not only with respect to their source and method of isolation, but also to reactivity. From the standpoint of specificity, the present antigens would appear to compare more closely to Campbell's 0.4 mole fraction antigen, but the two differ in that her antigen failed to react with serum taken early in the disease. A comparative study with human sera is planned.

Summary. A method was described for the isolation of antigens from the supernates of *H. capsulatum* yeast phase cultures by precipitation with zinc and alcohol accompanied by removal of protein by the water-chloroform method. Antigens prepared by this method fixed complement only with sera from rabbits infected with *H. capsulatum.* The antigens reacted with serum from both early and late bleedings of Histoplasma-infected rabbits, but failed to react with serum from animals with *B. dermatitidis, C. immitis, S. schenckii,* and *C. albicans.*

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Acute Experimental Hemorrhagic Shock in the Dog Treated with Subcutaneous Hyaluronidase-Dextran Solution. (21375)

ROBERT O. BAUER, WILLIAM H. GRAVES, JR., CHARLES S. HILL, AND LAWRENCE J. MILCH.

From the Department of Pharmacology and Biochemistry, USAF School of Aviation Medicine, Randolph Air Force Base, Randolph Field, Texas.

Dextrans, injected subcutaneously with hyaluronidase, have been demonstrated in the thoracic lymph of normovolemic cats and dogs (1,2). Dotti and Pyles(3) reported significant amounts of dextrans in venous blood of normovolemic rabbits one hour following subcutaneous dextran-hyaluronidase administration. Semple(4) demonstrated that an otherwise irreversible hemorrhagic shock in the dog may be alleviated by early intravenous injection of dextran.

It was the purpose of this work to determine

if similarly prepared hypovolemic animals (4,5) would survive following early subcutaneous administration of either dextran hyaluronidase physiologic saline (DSH) or hyaluronidase physiologic saline (PSH). Subcutaneous replacement therapy might have significant importance after national catastrophes when hemorrhage from blast injury might be expected in large numbers of the population. Inexperienced hands, under emergency recruitment, would find intravenous administration difficult, if not impossible.

Materials and methods. Mongrel dogs weighing 5.3 to 19.1 kg were anesthetized with 6% pentobarbital sodium solution, intravenously to loss of deep pain. Animals were exsanguinated to respiratory arrest using Semple's modification(4,5) of Walcott's controlled bleeding technic(6). At 60 to 90 seconds following cessation of respiration, intra-arterial reinfusion of hemorrhaged blood was begun. Within 1 to 2 minutes, 25% of blood volume previously removed was replaced. Control group of animals received no further treatment. A second group (DSH) received 6% dextran* in physiologic sodium chloride solution containing 300 TRU's%† of hyaluronidase. A final group (PSH) received hyaluronidase-saline solution alone. The infused volumes of DSH or PSH were equal to 75% of blood previously removed and administered rapidly (2-5 minutes) with the aid of 35 lb/sq. inch pressure into multiple subcutaneous sites. Blood pressure, heart rate, and respiratory rates were recorded kymographically in all animals before, during and for 30 minutes after completion of the treatment. In 3 DSH-treated dogs, venous blood levels of dextran were determined(7) at 4 and 8 hours post-treatment, and were 7 mg % (2 dogs) and 9 mg % (1 dog), respectively. All animals were returned to their cages and the 24-hour survival rate observed. The pertinent data are summarized in Table I.

TABLE I. Summary of Data Obtained from Dogs Subjected to Acute Hemorrhagic Shock and Treated with PSH or DSH by the Subcutaneous Route.

	Control	PSH	DSH
Wt, kg	9.4± 3.2*	10.4± 4.4	10.4± 3.6
Hemorrhage, cc/kg	49.2±16.4	52.8±20.8	47.5±17.1
Blood replaced, cc/kg	12.9± 3.9	13.5± 5.5	11.9± 5.5
Subcutaneous replacement, cc/kg	0	40.3±15.5	36.3±13.6
No. tested	24	27	28
No. survived	12	25†	13

* Mean wt and stand. dev. $\sqrt{\frac{\Sigma (y-\bar{y})^2}{n-1}}$.

† By "chi square" test survival in the PSH group is significantly higher ($P = <.01$) than survival in control or DSH groups; the latter pair are not significantly different.

Discussion. Data in Table I indicate that 93% of the PSH group survived, whereas only one-half of the dogs in the DSH or control group‡ were alive 24 hours following treatment. Contrariwise, Semple(5) found 17 out of 18 dogs survived following intravenously infused 6% dextran, 3 out of 8 following saline and 1 out of 8 controls. Failure of subcutaneous DSH to support survival is in agreement with the insignificant concentrations of dextran found in blood at 4 and 8 hours following infusions. Had the dextran entered the vascular system of the 28 dogs subcutaneously infused with DSH, almost complete survival presumedly could have been expected(4,5).

Subcutaneous administration of PSH apparently promotes survival in the dog following acute profuse hemorrhage. This survival might result from entrance of PSH into the plasma, increasing its volume. The immediate disappearance of PSH from injection site supports this suggestion. In contrast, however, persistent swelling and distention (at injection sites) lasting for some hours following DSH administration suggest fluid retention and ad-

* Commercially available material was supplied through the courtesy of Baxter Laboratories, Chicago, Ill.

† Turbidity-reducing units of Wyeth, hyaluronidase preparation "Wydase" used.

‡ The greater survival observed in the control group, undoubtedly resulted from free movement of unrestrained caged dog. Dr. Semple assures us that both he and Dr. Walcott restrained their animals on the table during the entire period of observation and that this restraint contributed to the marked discrepancy.

ditional decrease in blood volume. Minimal quantities of dextran detected as late as 8 hours following large amounts of DSH infused, suggest that most of this hydrophilic nonelectrolyte is retained at the injection site.

Summary. Seventy-nine dogs were exsanguinated to respiratory arrest and, after 60 to 90 seconds, reinfused with 25% of hemorrhaged blood. Twenty-four control dogs received no further treatment and half of this number survived 24 hours. Physiologic sodium chloride solution with hyaluronidase (PSH) was administered subcutaneously to 27 animals and 6% dextran in physiologic sodium chloride-hyaluronidase (DSH) to the remaining 28. 93% of the dogs treated with physiological saline-hyaluronidase were alive after 24 hours, whereas only 50% survival was recorded for the dextran group.

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Chlorpromazine and Tissue Metabolism. (21376)

MURRAY FINKELSTEIN, WILLIAM A. SPENCER, AND E. R. RIDGEWAY.
(Introduced by L. Swell.)

From the Research Laboratories, Veterans Administration Center, Martinsburg, W. Va.

We have indicated(1) that the effects of chlorpromazine* on the cardiovascular system are partially due to its direct action on the myocardium. In other studies on the mechanism of action, the *in vitro* effect of chlorpromazine on cardiac and brain tissue metabolism was examined. In view of recent reports(2,3) attributing pharmacological properties of chlorpromazine to a depression of brain oxygen consumption, we wish to report our observations.

Methods and materials. The oxygen consumption of cat heart left ventricle slices and brain cortex slices and homogenates was measured by the Warburg manometric technic; details in previous reports(4,5). Each experiment in duplicate or triplicate was repeated 5 times with heart slices and 4 times with either brain slices or homogenates, and when feasible, all doses were employed in each experiment. Chlorpromazine was added 2 hours, 1 hour or ½ hour after beginning of measured respiration of heart slices, brain slices or homogenates, respectively.

Results. Average results of typical experiments employing cerebral cortex slices are summarized in Fig. 1. For the sake of clarity only representative concentrations of chlorpromazine are presented. In concentrations

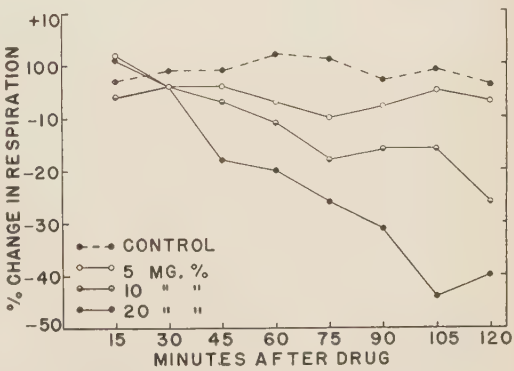

FIG. 1. Effect of concentration of chlorpromazine on respiration of slices of cat brain cortex.

* Generously supplied as "Thorazine" by Smith, Kline and French Laboratories, Philadelphia, Pa.; originally developed in France by Rhône-Poulenc.

of 5 mg% or less, there was essentially no effect, and randomly selected differences analyzed by the "t" test, indicated no significant variations throughout. Only in concentrations of 10 mg% and up was there a significant effect. Similarly, chlorpromazine concentrations to 5 mg% were without effect on left ventricle heart slices. However, concentrations of 10 and 20 mg% produced an average depression of approximately 45 and 90%, respectively, within 90 to 120 minutes. Results obtained with homogenized brain, and a single experiment with heart homogenate, were similar to those with slices.

Discussion. The response of patients and intact animals to relatively small doses of chlorpromazine (150 mg *per os* in humans, 0.5 to 5 mg/kg intravenously in dogs and cats) is considerable. Concentrations which were effective in experiments with isolated papillary muscle of cat heart(1) were from 0.025 to 0.5 mg%. A concentration of 1 mg% produced complete failure in contraction within 30 to 60 minutes(6). The present studies show that similar concentrations were without effect on the oxygen consumption of brain and cardiac tissue. This suggests, therefore, that the reported(2,3) fall in body temperature or total body metabolism following chlorpromazine administration is in all probability not due to a depression of brain oxidative metabolism. However, this does not preclude the possibility that the reduction in basal metabolism is due to a depressor effect on the central nervous system. Since the main energy source for activity of mammalian heart muscle is believed to be aerobic metabolism, it appears unlikely that therapeutic doses of chlorpromazine act on the heart by decreasing the utilization and/or production of energy. The possibility that chlorpromazine may affect this organ, and also the brain, by influencing sources of energy other than aerobic metabolism is not excluded.

In their demonstration of chlorpromazine depression of brain oxygen consumption, Courvoisier *et al.*(2), employing guinea pig cerebral cortex, used concentrations of 17.5, 35 and 70 mg%. Peruzzo and Forni(3), employing rat cerebral cortex, used concentrations of 2, 8 and 25 γ per 0.1 μl of suspension fluid. If plasma levels comparable to these relatively high *in vitro* concentrations are necessary to produce a depression of brain oxygen consumption *in vivo,* it would appear that, based on the plasma levels reported by Dubost and Pascal(7), massive intravenous doses would have to be administered.

Summary. Cat left ventricle heart slices and brain cortex slices and homogenates were employed in the Warburg manometric technic to examine the influence of chlorpromazine on their aerobic metabolism. Concentrations of the compound required to suppress their oxygen consumption were considerably greater than those that evoke a response *in vivo* and in other *in vitro* experiments. It appears unlikely that the pharmacological actions of chlorpromazine are due to an effect on oxidative metabolism.

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Peripheral Antagonism Between Hydrocortisone and Compound S.* (21377)

T. H. Rindani. (Introduced by M. C. Nath.)

From the Department of Physiology, Topiwala National Medical College, Bombay, India.

The marked antiinflammatory effect of topically applied hydrocortisone has been established. Using a special method described as the 'granuloma pouch' technic, Selye(1) demonstrated that administration of a very small amount of hydrocortisone in the cavity of the pouch caused an almost complete inhibition of the inflammatory reaction. The effect of topical application of several steroids on inflammation also shows that while hydrocortisone is antiinflammatory in action, DCA and other mineralocorticoids are generally proinflammatory(2). Since the granuloma pouch technic gives us a simple and yet an objective means of studying the local effects of the hormones on target tissue, an attempt was made to observe the effects of simultaneous administration of hydrocortisone and Compound S on inflammation, using this technic.

Procedure. 100 female Wistar rats, weighing between 120-140 g, were divided in 10 equal groups. Animals were kept 5 in a cage and allowed food and drink freely. Granuloma pouches were made according to the technic described by Selye(3), using 0.5 ml of 0.25% croton oil in corn oil as the irritant. On third day of experiment, varying doses of hydrocortisone acetate, Compound S and the combination of the 2 hormones were injected into the cavities of the pouches of the animals. Group I acting as control received 0.2 ml of suspending agent only. Microcrystalline suspensions of the steroids were made, using water containing Tween-80 as the suspending agent in such a way that 0.2 ml of the solution always contained the total amount of the hormone injected. All animals were sacrificed on tenth day of experiment and the exudate from granuloma pouch removed and measured. The results are summarized in Table I. The granulomas were dissected out, fixed in Suza solution and histological examination was done using hamalum eosin stains.

Results. As will be seen from Table I, hydrocortisone at dose level of 0.5 mg effectively reduces the amount of inflammatory exudate while Compound S in amounts of 5 to 10 mg moderately increases the same. Given in combination with 0.5 mg hydrocortisone however, the Compound S fails to increase the exudate, the average amount of which is actually reduced to about the same level as that obtained in animals treated with hydrocortisone alone. It thus appears that hydrocortisone even in smaller doses is able to assert its antiinflammatory action even in the presence of Compound S.

Histological examination confirmed the above findings. Hydrocortisone markedly inhibited and Compound S stimulated, the formation of granulation tissue. In animals treated with combination of the two, the amount and character of the granulation tissue were about the same as in those treated with hydrocortisone alone.

Profound depression of cellular reactivity by hydrocortisone seems to render the tissue less capable of reaction to irritation even in

TABLE I.
Effect of Hydrocortisone, Compound S and Their Combination on Amount of Exudate.

	Control	Hydrocortisone			Compound S			.5 mg hydrocortisone plus Compound S		
		100 γ	.5 mg	1 mg	1 mg	5 mg	10 mg	1 mg	5 mg	10 mg
Avg amt of exudate (ml)	17 ±2.3*	18 ±1.5	9.7 ±3.3	5 ±.97	15 ±2	23 ±1.9	24 ±.65	10 ±1.9	11 ±2.2	9 ±.95

* Stand. error.

* This work was carried out in part at the Institute of Experimental Medicine and Surgery, University of Montreal, Canada.

the presence of a proinflammatory hormone.

Summary. The effect of hydrocortisone and Compound S on inflammation, when administered locally, has been studied, using the 'Granuloma Pouch' technic described by Selye. Used separately, hydrocortisone suppresses inflammatory reaction while Compound S shows proinflammatory action. When both these hormones are administered simultaneously, however, hydrocortisone manifests its antiinflammatory action even in the presence of Compound S.

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Collagen and Hexosamine Content of Femurs of Rats.* (21378)

HARRY SOBEL, JESSIE MARMORSTON, AND FREDERICK J. MOORE.

From the Department of Biochemistry, Institute for Medical Research, Cedars of Lebanon Hospital, and the University of Southern California School of Medicine, Los Angeles.

Very little is known about the rates of deposition of collagen and mucopolysaccharides in bone during growth. This communication reports the results of analysis of the femurs of young rats for collagen and hexosamine. The latter substance is a constituent of mucopolysaccharides of bone.

It was found that the rate of deposition of collagen decreases somewhat as the animal grows. However, the rate of deposition of the hexosamine - containing material decreases markedly so that the ratio of hexosamine to collagen falls during growth.

Methods. Preparation of the femurs. The femurs were cleaned of all adhering muscle and connective tissue and extracted in a Soxhlet apparatus with a 1:1 mixture of acetone and ether to remove fat. The dried bones were subjected to a final cleaning and weighed. The length of each femur was uniformly measured with a vernier caliper. *Collagen estimation.* One femur was treated overnight with 10 cc of 10% trichloroacetic acid solution. This process was repeated with 5 cc of this solution for 12 hours, and finally with 20 cc of a mixture containing 85% acetone and 15% of the 10% trichloroacetic acid solution for 12 hours. The demineralized bones were then split lengthwise and dried. The marrow was scraped out as carefully as possible and discarded, and the weight of the organic residue of the bone was obtained. The residue was put into a 15 cc centrifuge tube and autoclaved with 3 cc of water for 4 hours at 15 lb pressure. The volume was adjusted to 15 cc and 2 cc of the supernatant fluid were taken for protein analysis using the biuret reagent(2) and employing Difco gelatin as a standard. *Hexosamine estimation.* For the determination of the total hexosamine content the other femur was treated with 5 cc of 3 N H_2SO_4 and autoclaved for 4 hours at 15 lb pressure. The volume was made up to 15 cc and 1 cc was taken for hexosamine analysis which was carried out by a modified Elson-Morgan procedure(3). The amounts of hexosamine and collagen were calculated for the quantity in both femurs. The rats were of the Addis-Slonaker strain and were maintained on stock diet(4).

Results. The analyses were carried out on the femurs of 57 male and 28 female rats from one to three months of age (the results are shown in Table I). Regression equations(5) were derived relating the quantity of these constituents to body weight and to combined femur length (Table II). The log-log relationships appeared linear. The femur length is a function of maximum growth attained by the animal and may be more useful than body weight in certain experimental situations in-

* This project was supported by a grant from the Public Health Service.

volving changes in fat, protein or water metabolism(6). Equation 1 in Table II shows

TABLE I. Results of Analysis of Femurs for Collagen and Hexosamine Content. The length, collagen and hexosamine are calculated for the sum of both femurs.

Weight, g	Length, cm	Collagen, mg	Hexosamine, mg
	Males		
30	3.02	16.6	1.12
33	3.10	21.2	1.22
37	3.14	22.4	1.18
41	3.48	26.4	1.20
43	3.40	24.4	1.24
43	3.38	23.6	1.28
47	3.46	25.2	1.25
50	3.60	27.7	1.36
69	4.13	51.6	2.00
75	4.34	52.2	1.59
77	4.36	52.0	1.97
84	4.35	62.6	2.32
85	4.33	63.2	2.24
90	4.75	69.8	2.25
96	4.66	71.0	2.16
103	4.84	65.0	1.87
103	4.91	70.8	1.98
105	4.74	69.8	2.32
107	4.97	65.0	1.56
110	4.80	70.8	2.24
116	4.85	77.8	1.96
118	4.98	76.8	2.16
119	5.10	69.0	2.05
121	4.94	74.4	2.02
122	4.75	70.6	1.88
123	4.88	70.8	2.13
124	5.10	82.8	2.44
130	4.99	79.4	2.52
130	5.27	88.4	2.40
134	5.24	74.8	2.24
150	5.35	106.0	2.52
153	5.29	86.4	2.47
172	5.50	96.6	2.48
175	5.67	100.3	3.00
187	5.76	100.6	2.93
188	5.56	104.6	2.79
203	5.85	100.3	2.90
205	5.60	100.0	2.78
208	6.00	118.0	2.78
217	5.85	127.7	2.94
222	5.95	131.0	2.88
224	5.98	139.0	3.30
227	5.91	115.8	2.76
229	5.94	121.8	2.92
230	6.00	119.0	2.40
234	5.80	120.5	3.18
239	6.08	137.0	2.87
242	6.20	127.5	2.83
243	5.98	129.0	2.66
248	6.18	138.0	3.06
255	6.10	142.3	3.18
258	6.14	142.5	3.25
264	6.46	142.5	3.40
268	6.35	143.5	2.99
270	6.17	145.0	3.00
271	6.22	150.5	3.26
274	6.36	172.0	2.55

TABLE I (continued).

Weight, g	Length, cm	Collagen, mg	Hexosamine, mg
	Females		
62	3.96	47.0	1.56
63	3.98	49.0	1.96
75	4.52	53.6	1.96
76	4.35	63.0	2.20
91	4.57	63.2	2.28
100	4.62	69.8	2.52
138	5.33	96.9	2.43
147	5.35	101.5	2.38
150	5.39	96.0	2.34
157	5.46	93.0	2.44
161	6.00	123.0	2.36
162	5.62	115.0	2.92
164	5.53	100.1	2.64
166	5.55	118.0	2.70
167	5.54	95.0	2.44
170	5.60	95.6	2.55
172	5.53	105.0	2.26
177	5.76	118.0	2.40
179	5.62	104.2	2.67
182	5.60	112.0	2.42
182	5.70	126.2	2.78
182	5.70	109.0	2.42
184	5.73	103.4	2.21
185	5.77	115.2	2.42
190	5.75	115.0	2.69
190	5.79	115.5	2.75
198	5.83	118.0	3.06
200	5.79	122.6	2.74

the correlation between the logarithm of body weight and the logarithm of femur length. A sex difference was not observed. Because of the high correlation between log body weight and log femur length ($r = .985$), correlations involving femur length mirror those of body weight.

Sex differences were observed in the collagen content of the femurs. At 50 g the femurs of females contained 25% more collagen than that of the males. This difference decreased with increase in weight and at 250 g the male and female values were almost identical. It appears highly improbable that sex differences which occur before puberty would tend to disappear after maturity has been reached. Gonadectomy and the administration of testosterone and α-estradiol did not specifically influence the deposition of collagen. Sex differences were not observed in the collagen content of the skin(1). The rate of deposition of collagen in the femurs for both males and females decreased gradually as the animal grew from approximately .6 mg per g gain in body weight in 50 g rats to approximately .5 mg of

TABLE II. Regression Equations Showing Correlation between Body Weight (W), Combined Femur Length (L) and the Collagen (C) and Hexosamine (H) Contents.

C and H are in mg, W is in g, L in cm.

Equations derived from $a = \bar{y} - b\bar{x}$ where $\bar{y} = \frac{\Sigma y}{n}$ and $\bar{x} = \frac{\Sigma x}{n}$

n = No. of cases; $b = \frac{k}{SS_x}$ where $k = (\Sigma xy - \bar{x}\Sigma y)$ and $SS_x = \Sigma x^2 - \frac{(\Sigma x)^2}{n}$

r = correlation coefficient $= \frac{k}{SS_x SS_y}$; $SS_y = \Sigma y^2 - \frac{(\Sigma y)^2}{n}$

Vb = variance of $b = SS_{b/N}$ where $SS_b = SS_y - SS_R$ and $SS_R = kb$; $N = n - 1$.

Standard deviation of the regression coefficient $= \sqrt{V_b}$

P = probability of sex difference. Separate regression equations are listed only where significant sex differences are found. The data were obtained on material from 57 males and 28 females.

SS_x for Log W = 4.1612; $\overline{\text{Log W}}$ = 2.1051 for ♂
= .6470 = 2.1588 for ♀

Log L = .4466; $\overline{\text{Log L}}$ = .6991 for ♂
= .0700 = .7261 for ♀

				P
1.	Log L = .01726 + .32539 Log W ± .01209	Log W*	r = .985	.9
2a. ♂	" C = −.02848 + .90874 " W ± .02467	" W	r = .980	.02
b. ♀	" C = .27812 + .78633 " W ± .04118	" W	r = .966	
3a. ♂	" H = −.63400 + .46631 " W ± .02311	" W	r = .939	.01
b. ♀	" H = −.25158 + .29481 " W ± .04719	" W	r = .775	
4a. ♂	" C = −.07189 + 2.79843 " L ± .05555	" L	r = .989	.01
b. ♀	" C = .22880 + 2.4058 " L ± .1132	" L	r = .972	
5a. ♂	" H = −.64174 + 1.41520 " L ± .07318	" L	r = .933	.01
b. ♀	" H = −.24637 + .8693 " L ± .1494	" L	r = .752	

* Stand. dev. of regression coefficient.

collagen per g gain in body weight in 250 g rats.† On the contrary, in the skin the rate of deposition of collagen increases during growth(1).

The regressions for hexosamine also showed a significant male-female difference. At 50 g the females contained 23% more hexosamine than the males. At 250 g, however, the calculated value for the female was less than that of the male. It is possible that in this instance also the sex difference is artifactual. The rate of deposition of hexosamine decreases sharply as the weight of the animal increases. Thus in 50 g rats approximately .013 and .016 mg of hexosamine per gram of body weight gain were deposited in the femurs in males and females respectively. At 250 g these values were approximately .0057 and .0034. In the skin the rate of deposition of hexosamine is nearly proportional to the rate of gain in weight and sex differences do not occur(1).

Because of the different rates of deposition, the hexosamine-collagen ratio (H/C) decreases as the animal grows. For 50 g rats the ratio is .044 and .043 for males and females respectively. At 250 g the values were calculated to be .021 and .020 respectively. The fall in H/C during growth was observed in the skin also(1). However the H/C is approximately four times greater in the femurs than the skin at any given weight.

Discussion. The rate of deposition of collagen and hexosamine in the femurs differs from the rate of deposition of these substances in the skin. Collagen is deposited in the skin at an increasing rate during growth(1), but in the femurs the rate of deposition decreases

† Net increment of each constituent per g body weight gained in different weight ranges may be calculated directly from the regression equations or by the following:

$$\frac{d \log y}{dx} = \frac{d(a + b \log x)}{dx}; \quad \frac{dy}{y} = b\frac{dx}{x}; \quad \frac{dy}{dx} = b\left(\frac{y}{x}\right)$$

slightly as the animal grows.

Hexosamine containing material in the skin is deposited at a slightly increasing rate during growth(1) but is deposited at a decreasing rate in the femurs. The ratio of hexosamine to collagen (H/C) falls in both organs as the animal grows. It would be of interest to learn if the decrease in H/C is related to the aging process since the relative reduction of ground substance gel to the quantity of fibre may interfere with metabolic processes. Investigations are underway to learn if this process continues in older animals, if it occurs in other organs and other species including man.

Summary. The collagen and hexosamine contents of the femurs of growing rats were determined. Regression equations were derived upon the basis that there is a linear relationship between the logarithm of body weight and of femur length and the logarithm of the quantity of collagen and hexosamine. Rate of collagen deposition decreases somewhat as the animal grows. Rate of deposition of hexosamine-containing material, decreases markedly so that the ratio of hexosamine to collagen decreases with growth.

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Determination of Serum Aldolase.* (21379)

JEAN L. COOK AND ALEXANDER L. DOUNCE.

From the Division of Cancer Research, Department of Medicine, and the Department of Biochemistry, the University of Rochester School of Medicine and Dentistry, Rochester, N. Y.

Recent work in determining the aldolase activity of human serum has led us to an investigation of the experimental conditions which might be most suitable in assaying serum aldolase. Four principal methods are found in the literature for measuring the aldolase activity of animal material. Meyerhoff and Lohmann(1) measured the alkali-labile phosphate which appears as the result of cleavage of hexose diphosphate to triose phosphates by the action of aldolase. Warburg and Christian(2) measured spectrophotometrically the reduction of coenzyme I (DPN) which occurs when 3-phosphoglyceraldehyde, one of the 2 triose phosphates produced by aldolase activity, is allowed to react with DPN in the presence of added crystalline-3-phosphoglyceraldehyde dehydrogenase and arsenate. Dounce and Beyer(3) measured the triose phosphates directly by an adaptation of the Barker and Summersen(4) colorimetric procedure for lactic acid determination, while Sibley and Lehninger(5) also measured the triose phosphates colorimetrically after forming colored addition compounds of these substances with 2,4-dinitrophenylhydrazine.

The formation of alkali-labile phosphate might be adapted to clinical use, but possible interference from serum phosphatase action makes the use of this method seem inadvisable. The method of Warburg and Christian is too complicated for clinical use. Since the method of Dounce and Beyer(3,8) was quickly found to be inapplicable to human serum owing to the inconveniently high blank values, our work was chiefly confined to investigation of

* This investigation was supported by research grants from the National Cancer Institute of the National Institutes of Health, Public Health Service, and from the American Cancer Society.

TABLE I. Effect of Buffer, pH and Temperature on Aldolase Activity.

	Dystrophic human serum				Normal human serum				Normal rat serum			
	Buffer pH 7.2		Buffer pH 8.6		Buffer pH 7.2		Buffer pH 8.6		Buffer pH 7.2		Buffer pH 8.6	
Temp., °C	Tris.	Coll.	Tris.	Coll.	Tris.	Coll.	Tris.	Coll.	Tris.	Coll.	Tris.	Coll.
25°	28	19	26	10	5.4	4.6	7.6	4.2	60	53	74	60
38°	69	46	65	42	9.2	7.6	10.8	5.2	172	143	198	146
Q_{10}	2.1	2.1	2.2	3.4	1.3*	1.3*	1.1*	1.9*	2.2	2.1	2.2	2.1

* Readings so low that Q_{10} values are virtually meaningless.
Figures other than Q_{10} values represent activity in "aldolase units"/ml serum.
Tris. = Tris-hydroxymethyl aminomethane buffer; Coll. = Collidine buffer.

the method of Sibley and Lehninger, which has already been used by others in slightly modified form(6,7) and which it was felt would be suitable for work with human serum, provided that certain discrepancies in the literature concerning temperature and pH optima(3,8) could be resolved.

Experimental. Procedure for Determining Aldolase. The procedure was carried out as described by Sibley and Lehninger(5), but with certain modifications to be discussed subsequently. The sample to be assayed was added to a test tube containing 1 ml of buffer, 0.25 ml of FDP (fructose-1,6-diphosphate) solution, 0.25 ml of hydrazine solution and sufficient water to make the total volume 2.5 ml. The tube was then placed in a water bath of the desired temperature. Thirty minutes after the enzyme was added the reaction was stopped by the addition of 2.0 ml of 10% trichloracetic acid. Each sample was run in duplicate, together with the blank which differed only in that the FDP was not added until after the reaction had been stopped. Precipitated protein was removed by centrifugation, and the color was developed by incubation of alkalinized aliquots of the supernatant, with 2,4-dinitrophenylhydrazine as described by Sibley and Lehninger(5). In this work, we have used the Evelyn colorimeter with a 540 mμ filter, set with the transmission of the blank at 100%. The aldolase activity of the samples was expressed in terms of arbitrary units, reading from the straight-line graph relating the transmission of the reaction mixture as determined above to the experimentally determined transmission of known dilutions of crystalline rabbit muscle aldolase.

Reagents. Fructose-1,6-diphosphate, 0.05 M, was prepared from the commercially available barium salt (Schwarz Laboratories, New York), which appears to be adequate(5) in this procedure without further purification. Barium was removed from the solution of FDP by the addition of the required amount of Na_2SO_4. The filtered final solution of 0.05 M sodium-FDP was kept frozen until used. Hydrazine sulphate (Merck) was brought to the desired pH with NaOH and was diluted to make the concentration 0.56 M. Tris-hydroxymethyl aminomethane buffer was adjusted to the desired pH with HCl, diluted to a concentration of 0.1 M, and stored in the cold. To prepare the dinitrophenylhydrazine solution, 1 g of 2,4-dinitrophenylhydrazine was dissolved in 1 liter of 2 N HCl, and the solution was filtered.

Results. The method of Sibley and Lehninger was found to give a low blank reading, and in other respects appeared to be suitable for the determination of aldolase in human serum. Effort was therefore concentrated on an attempt to establish the optimal conditions for determining serum aldolase. Some experiments were also carried out with muscle and liver aldolase, as well as aldolase crystallized from rabbit muscle, in order to resolve the discrepancies in the literature previously mentioned concerning pH and temperature optima.

pH Optimum. With serum, only a small and inconsistent change of activity with pH was found between pH 7.2 and 8.6 (Table I). The pH-activity curve for the aldolase of homogenates of human (dystrophic) liver was also found to be rather flat between pH 7.2 and 8.3, agreeing with the observation reported by Dounce *et al.* for rat liver homoge-

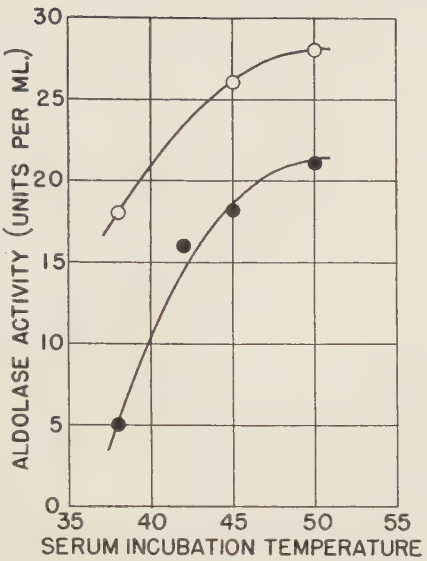

FIG. 1. Effect of temperature on aldolase activity of human serum using tris-hydroxymethyl aminomethane buffer at pH 7.2. ○ Serum of patient with progressive muscular dystrophy. ● Serum of patient with carcinoma of the prostate.

nate. In contrast, the pH optimum for crystallized rabbit muscle aldolase and the aldolase of human muscle homogenate (from a patient with progressive muscular dystrophy) was found to lie between 7 and 7.3. This agrees with the value reported by Dounce and co-workers for crystallized rabbit muscle aldolase and for the aldolase of rabbit muscle homogenate. Our assay method was that of Sibley and Lehninger, with tris-hydroxymethyl aminomethane buffer adjusted to the desired pH value.

Temperature Optimum. We have observed that the temperature optimum for human serum aldolase is in the neighborhood of 50°C, when the aldolase is measured with the method of Sibley and Lehninger at pH 7.2 using tris-hydroxymethyl aminomethane buffer (Fig. 1). The rate of the reaction is still increasing rapidly at 38°C, and since this temperature is safe for serum aldolase determination and also affords sufficient sensitivity, it has been adopted. The reason why serum aldolase has such a high temperature optimum is presumably that serum protein protects the aldolase against denaturation. Serum protein (Fig. 2), as well as the protein of homogenates and also egg albumin was found to protect crystalline rabbit muscle aldolase, which has a much lower temperature optimum than the aldolase in serum(8), against the effect of high temperature.

Effect of Buffer. Since the use of tris-hydroxymethyl aminomethane buffer leads to appreciably higher values for aldolase activity in human serum than does collidine buffer(3), (Table I), we have decided upon use of the former buffer in order to obtain as sensitive a reaction as possible. The addition of iodoacetate to block the action of 3-phosphoglyceraldehyde dehydrogenase has been found unnecessary in the assay of serum aldolase.

Conclusions. The method of Sibley and Lehninger is well suited to the determination of serum aldolase. We have altered the pH of the reaction mixture from 8.6 to 7.2, since this is close to the optimum for crystalline

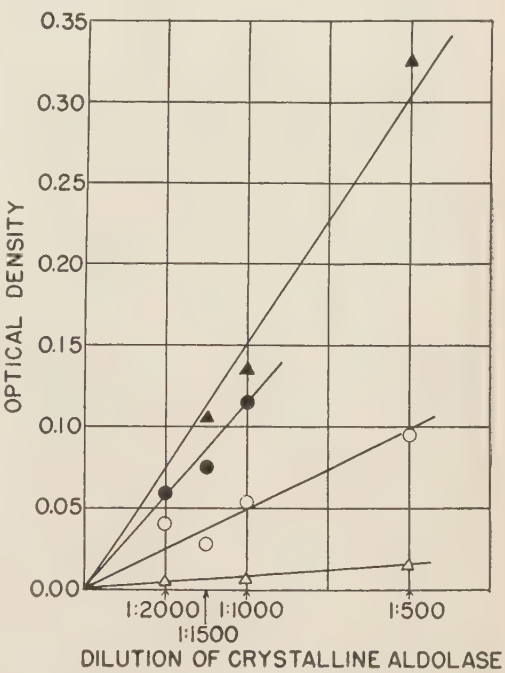

FIG. 2. Effect of serum protein on activity of crystalline rabbit muscle aldolase. ▲ Tris-hydroxymethyl aminomethane buffer, pH 8.6, 38°C, diluted in stored serum.* △ Tris-hydroxymethyl aminomethane buffer, pH 8.6, 38°C, diluted in water. ● Collidine buffer, pH 7.2, 25°, diluted in stored serum.* ○ Collidine buffer, pH 7.2, 25°, diluted in water.

* No aldolase activity present in serum.

aldolase of muscle and since the activity of human serum aldolase cannot be significantly increased by using a different pH. The possibility of interference from serum phosphatase is thus also decreased. It must be noted, however, that this method, unless further modified by lowering the temperature employed, is unsatisfactory for a comparative study of the aldolase of various tissues. Muscle aldolase in particular must be determined at a lower temperature in order to avoid partial destruction of the enzyme by heat. On the other hand, serum aldolase must be determined at a relatively high temperature in order to achieve sufficient sensitivity of the method, and as has been pointed out, this can be done without trouble owing to the relative resistance of the aldolase of serum to destruction by heat.

Two recent papers concerning aldolase should be mentioned. Leuthardt and Wolff (9) have found that there may be two different aldolases in animal tissue. If this is true, one might have an explanation for the two pH optima reported by Dounce and coworkers, and for differences in pH activity curves for crystalline aldolase and the aldolase in various tissue extracts.

Bruns(10) has recently reached conclusions in regard to the determination of serum aldolase which are not at great variance with ours, except that the use of collidine buffer is advocated by him. However, he seems not to have tried tris-hydroxymethyl aminomethane. Bruns has found an increase in serum aldolase in acute hepatitis and has made a valuable comparison of the serum aldolase activities of different animals and man.

Summary. Methods and conditions suitable for determining human serum aldolase have been studied. It is concluded that the method of Sibley and Lehninger, while not suitable without some modification for making comparative studies of aldolase of various tissues, is nevertheless well suited to the determination of serum aldolase, especially if the pH is altered to 7.2. Serum aldolase must be determined at a temperature of 38°C or higher in order to obtain a sufficient reaction rate for successful measurement, although this temperature is too high for the determination of crystalline aldolase or the aldolase of muscle homogenate.

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Degradation of Insulin-I^{131} by Liver and Kidney *in vivo*.* (21380)

NEIL J. ELGEE† AND ROBERT H. WILLIAMS.
From the Department of Medicine, University of Washington, Seattle.

It has been shown that insulin-I^{131} is rapidly degraded in the body(1). In this study an attempt was made to ascertain the contribution of liver and kidney to this degradation. Interest in these two tissues was aroused when it was demonstrated that they are major sites of radioactivity concentration following insulin-I^{131} administration.

Methods. Radioactivity of insulin-I^{131} is pro-

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† U. S. Public Health Service Research Fellow.

tein-bound, as indicated by its precipitation by trichloracetic acid and its non-dialyzability. After injecting it into rats, however, much of the radioactivity in tissues and fluids is non-precipitable with trichloracetic acid and thereby represents degraded insulin-I^{131}. The extent of this degradation has been measured in tissue and fluid samples removed from rats 15 minutes after they received insulin-I^{131} intravenously. To define the role of kidney and liver in the degradation, this rate has been determined in nephrectomized and physiologically hepatectomized rats and compared with the rate in normal animals. The technics used have been previously described(1). Male Sprague-Dawley rats weighed 130 to 170 g. They were kept in a constant temperature room (80°F) and fed a standard ration. Seventeen hours before the experiment, food and water were replaced by 10% dextrose in 0.9% saline; for 2 hours immediately before the experiment this also was withheld.

A. Viscerectomy. The experiment proceeded as follows: Rats were first operated on through a midline abdominal incision under ether anesthesia. Then 5 operative procedures were performed on different groups of animals (see below). As soon as operative wound was closed, insulin-I^{131} was injected intravenously. Fifteen minutes later, again under anesthesia, the animals were sacrificed, and their blood and tissue radioactivity assayed. The operative procedures used were as follows: a) *Nephrectomy,* 6 rats were nephrectomized following ligation of renal vessels. b) *Controls,* 7 rats were subjected only to handling of the viscera before closure of incision. c) *"Physiological hepatectomy"* was done in 7 rats by modification of the method of Russell(2). The gut from the mid portion of stomach to the descending colon was tied off in a mass ligature across the mesentery, including also the blood supply to spleen and pancreas. The lower third of stomach and the first 2 cm of the duodenum were not included in the ligature. The portal vein, hepatic artery and common bile duct were then ligated. d) *Hepatectomy controls,* 6 animals underwent above procedure (c) except that portal triad was not ligated. e) *Hepatectomy and nephrectomy,* 7 rats underwent combined physiological hepatectomy and nephrectomy and were compared with 6 controls similar to group (b). Insulin-I^{131}‡ was injected into the tail vein in Exp. A, in doses of 0.004 mg, or approximately 0.1 unit, containing 10 μc of radioactivity, and dissolved in 0.5 ml of 0.9% saline. B. *Portal vein injection.* To further investigate degradation by liver, an attempt was made to perform studies with insulin-I^{131} in the rat, comparable to those of Weisberg *et al.*(3) in the dog, measuring insulin-I^{131} degradation rather than insulin inactivation. Insulin-I^{131} was injected into the portal vein in several groups of rats over a period of a few seconds, and in 2 groups, over a 5 minute period, and levels of bound and supernatant radioactivity in blood and muscle were determined 15 and 5 minutes thereafter respectively. Control rats received insulin-I^{131} via inferior vena cava.

The animals of all groups were sacrificed by exsanguination through the dorsal aorta. Specimens of liver, kidney, and gastrocnemius muscle were removed. Radioactivity in these tissues was measured as described previously(1), the main feature being the division of radioactivity into 2 components: (a) that component precipitated by trichloracetic acid (TCA), being similar in this respect in insulin-I^{131}, and (b) TCA supernatant component, consisting of degradation products of insulin-I^{131}. Concentration of radioactivity was calculated as per cent of total dose of radioactivity per g of tissue or per ml of blood. This was multiplied by body weight/100 to allow for variations in body weight. The final expression of concentration [T]/[B] then represented per cent dose/g x body wt./100. Values greater than 1 indicated that the concentration of radioactivity was greater than the initial total body concentration. The concentrations of radioactivity in tissue fractions of experimental group of animals were compared with concentrations in corresponding tissue fractions in control group and the differences analyzed statistically by determination of the p value. (p values for all observations are shown in Fig. 1 and 2).

‡ Highly purified crystalline insulin, 26 to 27 units /mg, was kindly supplied by Eli Lilly and Co. and iodinated at the Abbott Laboratories at Oak Ridge.

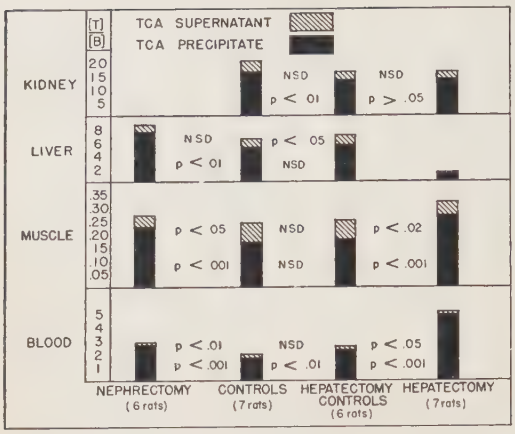

FIG. 1. Comparison of concentration, $\frac{[T]}{[B]}$, of radioactivity in tissue fractions 15 min. after intravenous insulin-I^{131} in rats with various organs removed (see text). Figures between columns are p values of differences between fractions in the 2 adjacent groups. NSD indicates no significant difference.

Results. A. *Viscerectomy.* All experiments were done 3 times with the same findings obtained consistently. Results of one experiment are reported here. Nephrectomy had a pronounced effect on degradation (Fig. 1). In blood, muscle, and liver, the concentration of protein-bound radioactivity was significantly increased over that in controls. Contrariwise, concentration of supernatant radioactivity—degraded insulin-I^{131}—was reduced in nephrectomized animals. It has been shown previously(1) that in normal rats, less than 2% of injected insulin-I^{131} radioactivity appears in the urine in 15 minutes, and that the majority of this radioactivity is not TCA precipitable. The finding, then, of less TCA supernatant radioactivity and more TCA precipitable radioactivity in the remaining tissues of nephrectomized rats, cannot be explained by the loss of renal excretory function. Recent work in this laboratory has demonstrated that insulin-I^{131} is degraded by kidney homogenates *in vitro* and the present data indicate that insulin-I^{131} is degraded less rapidly *in vivo* in the absence of the kidneys.

The same effect on distribution and degradation in muscle and blood resulted to an even greater degree after physiological hepatectomy (Fig. 1). Renal concentrations were not greatly affected. Since the procedure of physiological hepatectomy involved interruption of the blood supply of other abdominal viscera, the physiologically hepatectomized animals were compared in Fig. 1 to control animals who had interruption of the blood supply to these other viscera alone (so-called hepatectomy controls). These hepatectomy controls were further compared to the control animals who had laparotomy only, without ligation of visceral vessels. Bound radioactivity in the hepatectomy controls increased in the blood and decreased in the kidney but other fractions were not significantly altered.

When essentially all of the abdominal viscera (nephrectomy plus "hepatectomy") were removed from the circulation, blood and muscle levels of precipitable radioactivity were greatly increased (Fig. 2). However, 12.7% of blood radioactivity and 10.4% of muscle radioactivity was in the supernatant fraction, whereas less than 1% of the insulin-I^{131} injected was not TCA-precipitable. Some degradation therefore took place even in the absence of liver, kidney, and other abdominal viscera.

B. *Portal vein injection.* Of the several experiments to determine whether there was a difference in insulin-I^{131} degradation when it was injected into the portal vein as compared with injection into the vena cava, none showed

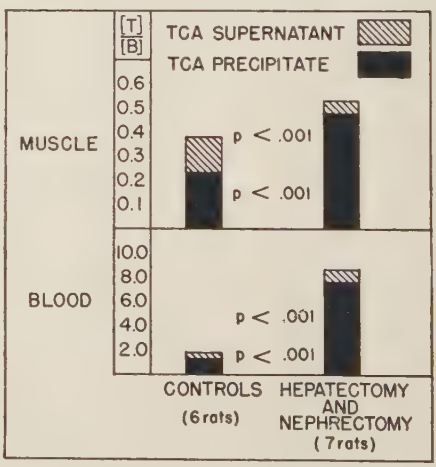

FIG. 2. Comparison of concentration, $\frac{[T]}{[B]}$, of radioactivity in tissue fractions 15 min. after intravenous insulin-I^{131} in control rats and in nephrectomized, physiologically hepatectomized rats.

significant differences in concentrations in either fraction in muscle or blood. The only differences were that concentrations were higher in the liver following portal vein injection, and in the kidney following vena cava injection.

Discussion. Shmidt *et al.*(4) reported that insulin was inactivated by homogenates of rabbit tissues, liver being most destructive, and kidney showing considerable activity. Shmidt postulated that the inactivation process involved proteolytic degradation. More recently, Mirsky *et al.*(5,6) confirmed this finding of the inactivation of insulin by liver and kidney *in vitro* using rat tissue. Tomizawa *et al.*(7) have further demonstrated proteolytic degradation of insulin and insulin-I^{131} in rat liver preparations. The present study has provided evidence that insulin-I^{131} is degraded by liver and kidney *in vivo* in the rat. In the absence of either organ, degradation was lessened, with more protein-bound radioactivity being found in blood and muscle.

Weisberg(3) was able to show, in the dog, that insulin was less potent as a hypoglycemic agent when injected into the splenic vein than when injected into the femoral vein, and this he interpreted to mean that the liver inactivated insulin. We know of no similar data in the rat, and it should be noted that while "physiological hepatectomy" demonstrated that the liver plays a role in insulin-I^{131} degradation, attempts to demonstrate increased degradation when insulin-I^{131} was injected into the portal system in the rat were unsuccessful. These effects may relate to differences in animal size, insulin sensitivity, and of course, very active insulin-I^{131} degradation by rat kidney.

Tissues other than liver and kidney also caused degradation since supernatant radioactivity appeared when these organs and other abdominal viscera were tied off. Whether the viscera, other than liver and kidney, cause degradation is not clear; although bound radioactivity increased in the blood, it was lessened in the kidney and not altered in muscle, and supernatant fractions were unchanged. *In vitro* studies, performed in this laboratory, have shown degradation by rat muscle but not by rat blood.

There is considerable evidence that the degradation of insulin-I^{131} is representative of that of unlabeled insulin(1,7). As noted above, there is also evidence that degradation accompanies inactivation of insulin. Since liver and kidney are sites of this degradation, it would seem reasonable to expect diminution of this function in certain diseases of these organs. Such a phenomenon might result in less inactivation of insulin, and in the case of a diabetic, might result in an apparent increase in the potency of administered insulin, and a decrease in the insulin requirement. One could speculate that this may be the case in certain instances of intercapillary glomerulosclerosis and in certain hepatic disorders in diabetics. Both of these conditions have been found associated with diminishing insulin requirements in some diabetics.

Summary. Nephrectomy or physiological hepatectomy decreased the rate of degradation of insulin-I^{131} in rats. There was some degradation even when both operations had been performed. Kidney and liver therefore are major, but not the only, sites of insulin-I^{131} degradation in the rat.

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Potent Psittacosis Antigens Free of Anticomplementary Activity. (21381)

ELINOR WHITNEY AND GLADYS M. GNESH. (Introduced by Irving Gordon.)
From the Division of Laboratories and Research, N. Y. State Department of Health, Albany.

The recent marked increase in reported cases of psittacosis(1) undoubtedly reflects only a portion of the true incidence, since mild cases are difficult to identify without laboratory aids(2). The most practicable laboratory examination is the complement-fixation test. For best results antigen should be made from psittacosis rather than from lymphogranuloma venereum strains(3-5), but the supply of psittacosis antigen has been insufficient to meet the demand. We therefore undertook to make our own antigen and found that lyophilization eliminated virtually all anticomplementary activity without diminishing antigenic potency, which was unusually high in some preparations. We adhered strictly to the methods presented below, which are based on the principles outlined by Davis(6).

Materials and methods. The antigens were phenol-enhanced and made from either allantoic fluid or yolk sac of embryonated hens' eggs. *Strains of virus.* The strains were isolated from parakeets, strains 5410 and 5411 in 1952, and 5465 and 54119 in 1954. They were adapted by rapid serial yolk-sac passage of heavy (20-33% by weight) yolk-sac suspensions in 7-day-old eggs. The volume of inoculum was 0.2 ml. The diluent was broth-salt solution* containing 100 units of penicillin and 1000 μg of streptomycin per ml. Yolk sacs were harvested from infected eggs as soon as embryos appeared sluggish or moribund, and stored as 50% (by weight) suspensions at –50°C. When sufficiently adapted, strains were titrated by intracerebral inoculation of 0.03 ml of 10-fold dilutions in 8- to 12-g Albany mice. This gave a better index of the elementary body content than did impression films stained by Macchiavello's technic. *Preparation of allantoic fluid antigen.* It was considered important to use only well-adapted strains of high mouse LD_{50} titer that had been stored for no longer than one week. The yolk-sac suspension was diluted to 15 or 20% (by weight), and 0.2 ml was inoculated into the allantoic sac of 10-day-old eggs. They were candled twice daily. Dead eggs were discarded. When the majority of embryos appeared sluggish or moribund, usually approximately 72 hours after inoculation, all the eggs were refrigerated overnight at 4°-6°C. Allantoic fluid was harvested in a number of small pools. Fluids contaminated with blood or yolk were discarded. The pools were cultured in thioglycollate semisolid medium and on blood-agar plates and portions were titrated in mice as described above. 5% phenol was added to each pool in a final concentration of 1%. The flasks were stoppered tightly and stored at 4°-6°C for 10 days. They were shaken daily. Safety tests of undiluted phenolized fluid consisted of intracerebral inoculation in mice. A blind passage was done after one week, and all animals were observed for a month. Elementary bodies were concentrated by centrifugation in SSl Servall angle head at 5000 r.p.m. for one hour. The supernatant fluid was decanted and the sediment resuspended to one-fifth the original volume in cold isotonic solution of sodium chloride, phosphate buffered to pH 7.0(7a). 'Merthiolate' was added to a concentration of 1:10,000. Aliquots of the antigen were shell-frozen in a methanol-dry-ice bath, then lyophilized immediately, vacuum-sealed, and stored at 4°-6°C. Before use antigens were reconstituted with sterile distilled water to the original volume. *Preparation of yolk-sac antigen.* The technic used to make phenol-enhanced yolk-sac antigens differed from that above in the following respects: 7-day-old eggs were inoculated; yolk-sac suspension was made by the technic of Bedson *et al.*(5), with 2.0 ml of sterile phosphate buffer (pH 7.6) added per yolk sac. Prior to concentration large particles were removed by preliminary sedimentation hori-

* Broth-salt solution consists of 1 part beef-infusion broth and 9 parts of 0.85% sodium chloride solution adjusted to pH 8 before autoclaving. After sterilization pH is 7.4-7.6.

zontally at 1500 r.p.m. for 15 minutes in an International No. 1 centrifuge. Subsequent concentration at 5000 r.p.m. was done for 1½ hours instead of for one hour and the sediment was taken up with cold buffered saline to one-half instead of one-fifth the original volume. After reconstitution it was found necessary to clarify yolk-sac antigen before use since it usually contained powdery debris that interfered with reading in complement-fixation tests. This was accomplished by horizontal centrifugation at 1000 r.p.m. for 5 minutes. *Control antigens* were prepared from uninfected eggs. Allantoic fluid or yolk-sac suspension was processed as described. *Complement-fixation tests.* The technic of Wadsworth, Maltaner, and Maltaner(7b,8) was modified to suit the circumstances. Sera were inactivated at 60°C for 30 minutes. The fixation period was 24 hours at 4°-6°C. Optimum antigen dose was determined in the presence of three 50% complement units. One-tenth milliliter of optimum dilution gave maximum specific fixation with no anticomplementary activity. Each antigen dilution was tested for anticomplementary properties with one, two, and three 50% units of complement. Dilutions that gave less than 90% hemolysis with 2 units were considered anticomplementary.

Results. It was found that highly potent antigens free of anti-complementary activity could be made from 2 of the 4 recently isolated strains (Table I). These preparations gave optimum fixation of complement at dilutions of 1:64 in tests with serum of moderate titer obtained during convalescence from a patient with psittacosis. Although other preparations were not as potent, several of them (5465 YS_7, 5410 M_1YS_{16} and M_1YS_{15}, and 54119 $YS_3M_1YS_5$) could be used in lower dilutions often employed.

In spite of the fact that the lyophilized preparations were reconstituted to volumes less than those of the original fluids or tissue suspensions, they exhibited no detectable anticomplementary properties. The lyophilized preparations kept well for 4 months, the longest period tested at present. Several anticomplementary liquid antigens were sufficient-

TABLE I. Potency and Stability of Liquid and Lyophilized Psittacosis Yolk-Sac and Allantoic Fluid Antigens in Complement-Fixation Tests with a Single Human Convalescent Psittacosis Serum.

Strain No.	Passage level	Conc. of inoculum, %	Antigen source	Mouse titer (LD_{50})* Inoculum	Mouse titer (LD_{50})* Final antigen fluid	Storage (days)	Anticomplementary activity of antigen Liquid (dilution)†	Anticomplementary activity of antigen Lyophilized (dilution)§	Optimum dilution of antigen in CF tests Liquid	Optimum dilution of antigen in CF tests Lyophilized
5465	YS_7	20.0	AF	7.1	6.0	1	64	None undil.	Unsat.	8
5410	M_1YS_{16}	16.6	AF	6.5	6.3	4	4	None undil.	8	8
						96	4	None 4	8	8
	M_1YS_{16}	26.6	YS	6.5	7.0	12	256	None 4	Unsat.	64
						82	32	None 16	,,	64
	M_1YS_{17}‖	16.0	AF	ND	6.0	12	16	2‡	32	64
						55	None 32§	None 64	32	64
	M_1YS_{15}¶	16.0	AF	6.5	4.7	8	ND	None 4	ND	8
5411	YS_{17}	10.0	AF	6.0	5.5	4	Undil.	None undil.	2	2
						98	Undil.	None undil.	2	Undil.
	YS_{16}	16.0	YS	ND	6.1	12	64	None 4	Unsat.	64
						82	64	None 64	,,	64
						122	ND	None 32	ND	64
54119	$YS_3M_1YS_5$	20.0	AF	5.75	5.8	5	ND	None undil.	ND	4

YS = Yolk sac. AF = Allantoic fluid. M = Mouse. Undil. = Undiluted. CF = Complement-fixation. Unsat. = Unsatisfactory because anticomplementary. ND = Not done.

* Reciprocal of the log dilution LD_{50} calculated by moving avg interpolation method(9). † Reciprocal of dilution tested. ‡ Reciprocal of dilution in which anticomplementary activity is still present. § Reciprocal of lowest dilution tested. ‖ Lot 71. ¶ Pullets' eggs used.

ly potent so that the optimum dilution fell beyond the anticomplementary range. While they could have been used, they were obviously inferior to the lyophilized aliquots of the same lots.

The best antigens were made from inocula that had high mouse infectivity titers and had been carried through 16 or more rapid yolk-sac transfers. Some of the less potent antigens, however, were also made from inocula that had high mouse LD_{50} titers and had been through 16 rapid passages, so these are not the only factors responsible for the good results.

A limited number of tests with human, ferret, or guinea pig sera containing antibodies against other viruses or against rickettsial agents showed that the psittacosis antigen had little or no nonspecific activity except for expected cross reactions with other members of the psittacosis-lymphogranuloma venereum group. In only 2 of the other sera examined was any activity noted. A serum from a patient convalescent from Rocky Mountain spotted fever had the low titer of 11, and a ferret anti-mumps serum gave partial fixation, having an atypical pattern in 1:8 and 1:16 dilutions. The following antisera gave no reaction: Influenza viruses Type A, B, and C; lymphocytic choriomeningitis; St. Louis encephalitis; herpes simplex; Eastern and Western equine encephalomyelitis; epidemic and murine typhus; and Q fever. Psittacosis antigen 71 (Table I) gave consistently higher titers than a lymphogranuloma venereum antigen ("Lygranum"†) not only in repeated tests with a single psittacosis convalescent serum, but also with serum of a patient convalescent from lymphogranuloma venereum (Fig. 1). The titer of the lymphogranuloma venereum serum was ~ 161 in all but a single test with psittacosis antigen 71, but varied considerably in simultaneous tests with Lygranum antigen. The sera were examined repeatedly as positive controls with these antigens over a period of several weeks.

One hundred ninety-eight sera submitted for diagnosis and 100 sera from healthy persons were also examined with both psittacosis antigen 71 and Lygranum. Thirty-one single specimens reacted. In addition, multiple bleedings were obtained from each of 19 patients. In both groups the psittacosis antigen gave higher titers than Lygranum (Fig. 2 and Table II), and in the case of the acute- and convalescent-phase serum pairs, higher rises in titer (Table II); but in no case did the psittacosis antigen detect a rise in titer not also shown by Lygranum.

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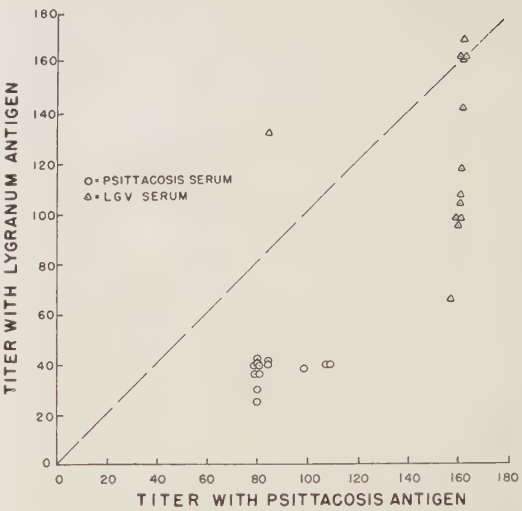

FIG. 1. Repeated titrations of 2 sera with psittacosis and Lygranum antigens.

Discussion. Heavy inoculum is necessary to make potent allantoic fluid psittacosis antigen, because it insures rapid multiplication (10) and sufficient lecithin carry-over from the infected yolk sac to potentiate the antigen (6). This is true not only of the psittacosis group but also for the rickettsiae(11). The antigenic content of infected allantoic or yolk sacs appears to be at its maximum when embryos are moribund. After they die the amount of antigen may diminish so that it is important to candle infected eggs repeatedly and harvest just before the embryos die. If the inoculum fails to kill the embryos by the end of the 3rd day, antigen from that group of eggs is likely to be weak(4,12).

Rapid serial passage of recently isolated strains seems to step up the virulence of psittacosis strains. Our data are insufficient, however, to determine precisely the factors responsible for the antigen potency. Our method is

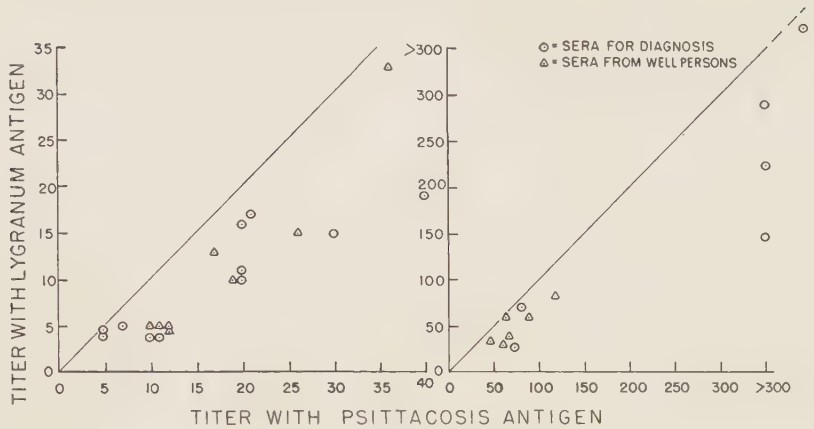

FIG. 2. Titrations with psittacosis and Lygranum antigens. Sera from 17 patients, 14 well persons.

empirical and closely follows principles laid down by Meyer and his co-workers(12), Smadel *et al.*(4), and Davis(6).

The effectiveness of lyophilization in removing anticomplementary activity from liquid antigen was striking. To determine whether this effect is related to the mechanism by which dialysis removes anticomplementary activity(6) must be the subject of future work. Lyophilized antigens are stable for 4 months, the longest period tested. Whether they will prove as stable as the ether-extract antigens studied by Hilleman and Nigg(13) and Smadel(4) remains to be learned. Bedson *et al.* (5) found that their refrigerated psittacosis antigens were good for several months. Meyer and Eddie(14) exhausted their antigens within a relatively short time after they were produced; in fact, this has been the case with many recent psittacosis antigens. The prac-

TABLE II. Complement-Fixation Titers with Psittacosis and Lygranum Antigens of Multiple Serum Specimens from Each of 19 Patients.

Clinical data	Psittacosis antigen No. 71 — Titers* a	b	c	d	Max. fold rise	Lygranum — Titers* a	b	c	d	Max. fold rise
P, S	26	>216	>216	104	8.3	16	88	104	46	6
P, S	<4	AC	66	20	16.5	<4	AC	33	16	8
P, S	<4	88	108		27	<4	46	56		14
P, S	5	72	71		14.4	4	38	42		10.5
P, S	AC	40	40		0	AC	21	18		0
P, S	4	72			18	4	40			10
P, S	<16	72			4.5	<16	59			3.6
P, S	643	>1285			2	415	676			1.6
0	11	14			0	4	8			2
HI†	50	52			0	33	36			0
HI†	643	321			0	475	236			0
HI	<4	40			10	<4	16			4
HI	21	14			0	17	11			0
S	132	132			0	81	81			0
S	84	84			0	59	44			0
P, S	7	80			11	<4	40			10
P, S	>321	>321			0	145	192			1.3
P, S	8	5			0	10	10			0

P = Contact with parakeet. S = Symptoms and signs consistent with psittacosis. 0 (under clinical data) = No contact with parakeet, mild illness. HI = History of case incomplete. AC = Anticomplementary serum.

* Calculated No. of 50% complement units that would be fixed by undiluted serum; antibody content considered low if titer <20, very low if titer <10.

† We wish to thank Drs. K. F. Meyer and B. Eddie for these specimens.

tical usefulness of lyophilization may be to remove anticomplementary activity rather than to increase stability on storage.

Summary. A method for the preparation of psittacosis antigen of satisfactory potency is presented. When lyophilized, the antigen is free of anticomplementary activity, stable for the 4-month period tested, apparently more sensitive than a lymphogranuloma venereum antigen in the detection of psittacosis antibodies, and apparently free of nonspecific properties.

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Failure of Protein to Protect Against Cholesterol Atherogenesis in Underfed Rabbits. (21382)

LEO LOEWE, MARTIN G. GOLDNER, STANLEY M. RAPOPORT, AND ILLA STERN.

From the Jacques Loewe Research Foundation Laboratories, Jewish Hospital of Brooklyn, and the Jewish Chronic Disease Hospital, Brooklyn, N. Y

In previous experiments we have shown that cholesterol atherosclerosis in rabbits is not inhibited by caloric restriction and that cholesterol induced elevations of blood lipid levels are even greater in rabbits on starvation diets than in well fed control animals(1). Since our restricted diet was low in all nutrients including protein, the question presented itself whether our results were due solely to caloric restriction or in part to deficiencies in protein and lipotropic factors. The role of protein and lipotropic agents in protection against development of atherosclerosis is by no means clear. Duff and Meissner(2) showed that even large amounts of choline had no inhibitory effect on cholesterol atherosclerosis in rabbits, while Li and Freeman(3) found that a practically protein free diet is necessary in order to induce hyperlipemia in the normal dog by cholesterol feeding. In thiouracil-treated dog, however, even a liberal protein diet does not prevent cholesterol atherogenesis, as demonstrated by Steiner and Kendall(4).

To investigate further this problem we have compared the effect of cholesterol feeding in 2 groups of rabbits, kept on diets of similar caloric restriction but of different protein contents. One diet was relatively rich in protein and lipotropic agents while the other was poor.

Material and methods. Male, adult, white Swiss rabbits weighing between 2.9 and 4.6 kg were used. The animals had been kept in the laboratory for several weeks prior to experiments, each on predetermined daily food intake to maintain weight equilibrium. Two groups of animals were then placed on one-third of the normal ration. These calorically restricted diets were set up so that Group A

had a restricted protein intake of 4.5 g/day and Group B about 10 g/day with a methionine and choline content twice as high as Group A. *Group A,* consisting of 13 rabbits, were fed 30 g/day of the following diet:* protein 16%, fat 3%, fiber 20%, methionine 0.33%, choline 490 mg/lb, and arginine 1.1%. *Group B,* 12 rabbits, were placed on an isocaloric but higher protein diet and received 30 g/day of the following diet:* protein 35%, fat 3%, fiber 9%, methionine 0.67%, choline 1,020 mg/lb, and arginine 2.4%. Each rabbit received in addition 2 g cholesterol/day fed with approximately 10-20 g of fresh carrots several hours prior to feeding of daily ration. These feedings were continued for 9 weeks, at which time all animals were sacrificed. Weights were determined at weekly intervals. The following biochemical determinations were made for each animal at the start of the experiments, during the fifth week, and at the end: 1. Total blood cholesterol (method of Abell, Levy, Brodie and Kendall) (5). 2. Blood phospholipids (method of Youngburg and Youngburg) (6). 3. Blood fatty acids (method of Stern and Shapiro) (7). 4. Blood lipoproteins (method of Gofman and associates) (8) were determined only at beginning and end of the experiment.† All tests were run in duplicate and an agreement within 3% was required. After the animals were sacrificed, their aortas were examined by gross and microscopic means. A grading system from one to 4+ was employed, where one plus indicated minimal involvement and 4+ indicated plaque formation and extensive involvement of the entire aorta. In the present paper only average data will be given.

Results: The results are represented in Table I.

Weight: Both groups showed marked weight reduction at an essentially parallel rate. Over the entire period, the group on low protein diet lost an average of 26.2%, whereas the high protein group lost 24%. In the former group the weight loss varied between 15.7 and 41.3%, in the latter from 16.5 to 38.7%.

TABLE I. Initial and Final Average Values of Body Weight, Blood Cholesterol, Phospholipids, Fatty Acids and Lipoproteins in 2 Groups of Rabbits Receiving Cholesterol and Isocaloric Diets with Different Protein Content.

Exp. group		Subnutrition, regular diet	Subnutrition, high protein diet
No. of rabbits		13	12
Duration of exp.		9 wk	9 wk
Cholesterol dose		2 g/day	2 g/day
Avg wt—initial		3.89 kg	3.96 kg
" " —final		2.87 "	2.98 "
" " change		1.02 "	0.98 "
Blood cholesterol—I		27.6 mg %	22.1 mg %
	F	1359.0 "	1254.0 "
Blood phospholipids—I		3.2 "	2.8 "
	F	16.6 "	14.3 "
Blood fatty acids—I		129 "	108 "
	F	617 "	563 "
Blood lipoproteins (mg %)			
Sf 0- 11	I	33.5	24.5
	F	241.0	234.0
11- 21	I	20.4	13.2
	F	501.0	572.0
21- 35	I	6.5	8.5
	F	626.0	565.0
35-100	I	15.4	18.2
	F	854.0	625.0
100-400	I	19.7	17.6
	F	605.0	483.0

Biochemical findings: Blood cholesterol showed a marked rise in both groups and reached values of 50 times normal level. Blood phospholipids and fatty acids rose in both groups to about 5 times the normal level. The various groups of lipoproteins increased by more than 50 times of the initial values. In general the values for the animals on subnutrition regular diet were somewhat higher than those on the subnutrition high protein diet. These differences, however, were not statistically significant, although they appeared to be rather marked particularly in the lipoproteins of flotation rates of 35-100 and above.

Anatomical findings: The aortas of all animals showed atherosclerotic involvement. Table II shows that lesions in the high protein group appeared to be even more marked than those in the regular subnutrition group.

Discussion: The results of this experiment

* Manufactured by Camp Milling Co., Walton, N. Y.

† Lipoprotein determinations were processed in the Viral and Rickettsial Research Laboratories, Lederle Laboratories Division, American Cyanamid Co, Pearl River, N. Y. We wish to express our gratitude to Dr. Raymond A. Brown for his cooperation.

TABLE II. Degree of Atherosclerotic Lesions in Groups A and B.

Group	4+	3-4+	3+	2-3+	2+	2-1+	1+
A	2	2	2	4	2	1	0
B	3	5	1	2	1	0	0

support the findings of previous experiments that cholesterol-fed rabbits on undernutrition regimen developed a marked atherosclerosis, together with a marked increase in blood lipids and lipoproteins. It now appears that variations in the protein content of the subnutrition diet do not affect the degree cholesterol atherogenesis. A cholesterol-fed group of rabbits maintained on a restricted diet containing a rather adequate amount of protein and lipotropic substances developed atherosclerotic and blood lipid changes of at least the same degree as a control group on the same caloric restriction with additional restrictions in proteins and lipotropic substances. Thus an adequate protein content of an otherwise caloricallly restricted diet did not exert any protective action.

Summary. 1. Study of effect of protein on development of experimental atherosclerosis on blood lipids and lipoproteins, in underfed rabbits with supplementary cholesterol, was performed. 2. Cholesterol fed rabbits on a limited intake of standard laboratory food lost approximately one-fourth of their body weight. A marked degree of atherosclerosis, increased blood lipids and lipoproteins was produced. 3. Cholesterol-fed rabbits on a limited intake of a relatively high protein diet, who lost equivalent amounts of weight, developed approximately the same degree of atherosclerosis, increase in blood lipids and lipoproteins.

The authors wish to express their appreciation to Mr. Paul J. Geller for his technical assistance.

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Electrophoresis of Plasma Proteins and Ascitic Fluid of Dogs with Radiation Cirrhosis.* (21383)

DORIS H. CLOUET, CON O. T. BALL, GEORGE R. MENEELY, AND PAUL F. HAHN.

From the Research Laboratory and Radioisotope Unit, Thayer Veterans Administration Hospital; Departments of Biochemistry, Preventive Medicine and Medicine, Vanderbilt University Medical School; and Cancer Research Laboratories, Meharry Medical College, Nashville, Tenn.

Elsewhere we demonstrated the production of cirrhosis and ascites in young dogs following repeated intravenous administration of large amounts of radioactive colloidal gold which delivered massive amounts of irradiation to the liver(1). A report of a more comprehensive study of such internally administered irradiation is being prepared(2). A synergistic effect is encountered when hemorrhage is superimposed on internal irradiation(3).

It seemed of interest to study the distribution of plasma proteins in some of these animals, and, therefore, electrophoretic patterns of plasma samples were made in certain cases, described below. When ascites was present proteins of ascitic fluid were examined

* This study was carried out in part with funds from the Josiah Macy Jr. Foundation.

TABLE I. Dog No. 52262.

Date	Bleeding (4200 ml total)	Intrav. Au^{198}	Supportive therapy penicillin units	Remarks
3/19–28	6 units of 200 ml ea.			
4/ 1		80 mc		
4/ 4–5/2	5 *Idem*			
5/ 5		46 mc		
5/11	200 ml			
5/23–25			300,000 ea.	
5/30–6/3	400 "			
6/ 3– 8			"	
6/ 9	200 "			
6/17	200 "			Clinical ascites by palpation
6/28–7/28	5 units of 200 ml ea.			
8/ 3				" " tapped 120 ml
8/ 5				Tapped 1400 ml fluid
8/14				" 2100 " "
8/15				" 2000 " " sacrificed

as well. The chief changes in plasma proteins of treated dogs were a decrease in albumin fraction and an increase in gamma globulin fraction. After development of clinical ascites the major component in ascitic fluids was albumin with $alpha_2$ globulin, fibrinogen and gamma globulin also appearing in most samples.

Methods. Dogs were kept under observation for at least several weeks before use. During this time they were dipped to remove external parasites, vaccinated for distemper, and de-wormed. They were fed Purina Dog Chow *ad lib.* In one instance litter mates were used to eliminate as far as possible individual variations in response to treatment. A typical protocol for one of these animals follows: Dog 52262, age approximately 1½ years, was a mongrel bitch acquired 9/15/52. She was maintained until 3/19/53 when bleeding was begun. All further treatment is recorded in Table I. On 8/15 the animal was sacrificed. The general appearance was normal with the exception of abdominal distention. About 2000 ml of clear fluid was aspirated before autopsy. A blood sample for electrophoretic analysis was withdrawn on 5/11/53 before ascites developed, and another on 8/5/53 after onset of ascites. Dogs which served as controls were under the same conditions as treated dogs. *Blood* was drawn into dry sodium oxalate, centrifuged, and plasma drawn off for electrophoretic studies. Similarly, ascitic fluid was drawn into dry sodium oxalate. *Plasma* was diluted with 3 volumes of veronal buffer, pH 8.6, of 0.1 ionic strength, and dialyzed for 24 hours before electrophoretic analysis. The ascitic fluid was examined in undiluted state. All samples were analyzed in a Klett Longsworth-Tiselius apparatus with veronal buffer described above. The patterns were obtained by scanning method after 3 hours in an average electric field of 4 volts/cm. Total nitrogen of each sample was determined by a semi-micro Kjeldahl method.

Results. In Table II are the results of electrophoretic analyses. Following the Table is a statistical analysis of values in the Table.

In Table III are the results of electrophoretic analyses of ascitic fluids. In these samples the average protein concentration was about 0.5 g %.

Among experimental animals there are 4 categories of treated dogs. In some instances several episodes of irradiation preceded bleeding. In others bleeding was begun first and was subsequently followed by irradiation.

Discussion. In electrophoretic studies of serum proteins in human hepatic cirrhosis with and without ascites a decrease in albumin was found by Gray and Barron(4) with the albumin ranging from 13.6 to 49.0 and averaging 29.3% of total protein with increase in the alpha- and beta-globulin components in several instances. Ricketts and his associates(5) found albumin diminished in cirrhosis alone and more severely when ascites was present.

TABLE II. Electrophoretic Fractions of Plasma Proteins. (As % of total: mean values ± SD.)

Group	Alb.	α_1 glob.	α_2 glob.	α_3 glob.	β glob.	Fib.*	γ glob.	A/G ratio
Control:								
8 adults								
1 puppy	37.8 ± 4.1	7.5 ± 1.5	5.1 ± 2.0	12.0 ± 1.7	10.5 ± 2.0	16.5 ± 3.6	9.9 ± 2.7	.85 ± .13
Bled, irradiated:								
No ascites								
3 adults	33.1 ± 2.2	8.0 ± 1.4	6.9 ± 0.7	9.6 ± 4.3	13.6 ± 3.0	19.5 ± 3.2	9.1 ± 2.2	.74 ± .06
Irradiated:								
Ascites present								
1 adult								
4 puppies†	24.7 ± 7.3	8.3 ± 0.6	8.2 ± 2.5	9.3 ± 3.3	12.9 ± 5.2	20.2 ± 4.3	16.7 ± 7.6	.46 ± .13
Bled, irradiated or irrad., bled:								
Ascites present								
4 adults								
1 puppy	21.5 ± 4.9	6.7 ± 2.4	8.0 ± 1.6	7.1 ± 2.2	13.4 ± 1.5	30.1 ± 3.1	13.4 ± 2.6	.47 ± .14

* Omitted from total globulin in A/G ratio. † 2 litter mates of control puppy.

Differences. The mean albumin fraction was highest in control group; significantly higher ($P < 0.01$) than in groups with ascites. Bled-irradiated dogs without ascites showed a significantly higher ($P < 0.01$) mean albumin fraction than dogs similarly treated developing ascites. The alpha$_1$ globulin fractions were not altered. The mean alpha$_2$ globulin fraction was significantly lower ($P < 0.05$) in controls than in groups with ascites. The mean alpha$_3$ globulin fraction was highest in control group and significantly higher ($P < 0.05$) than in bled-irradiated dogs with ascites. Mean beta globulin fraction was lowest in control group; but again differences were significant ($P < 0.02$) only in contrast to bled-irradiated group with ascites. Mean fibrinogen fraction was lowest in control group; significantly lower ($P < 0.001$) than in bled-irradiated group with ascites. Fibrinogen of this latter group was significantly higher ($P < 0.01$) than both similarly treated group without ascites and irradiated group with ascites. Mean gamma globulin fraction of control group was significantly lower than irradiated group with ascites ($P < 0.05$) and the irradiated-bled group with ascites ($P < 0.001$). Mean A/G ratio was highest in control group; significantly higher ($P < 0.001$) than both irradiated dogs with ascites and irradiated-bled group with ascites. Mean of bled-irradiated group without ascites was significantly higher ($P < 0.01$) than both similarly treated dogs with ascites and irradiated group with ascites.

The alpha$_1$ and alpha$_2$ globulins were not much affected but the beta and gamma globulins were increased and the latter more so when ascites was present.

As pointed out(1,2) there is apparently an important age factor which exerts some influence over production of ascites by internal irradiation under conditions used in these experiments. When sufficient irradiation (40,000 to 80,000 equivalent beta roentgens as calculated) is delivered to the hepatic region of young pups(6), provided one waits a sufficient period of time (50-100 days), it has quite uniformly been possible to produce cirrhosis and ascites. A considerable number of older animals were irradiated by the same procedures and with approximately the same number of equivalent beta roentgens to the liver without production of ascites detectable by external examination or aspiration(2).

As shown in Table II the most uniform differences between irradiated and control groups of animals were that plasma albumins were lowered significantly in the former group. On the other hand, irradiated animals showed an increase in gamma globulin fraction. There also appeared to be an increase in fibrinogen in the two groups in which ascites developed.

Protein fractions with the mobility of each of the plasma proteins were found in one or more of the ascitic fluid samples. Albumin, alpha$_2$ globulin, fibrinogen and gamma globulin appeared in highest concentrations in these samples.

There was considerable variation in time required for onset of ascites as well as total amount of irradiation necessary for changes in the liver to give rise to this condition. The levels and frequency of dosage of colloidal gold are obviously important variables but as indicated, there must be other factors.

The data are not complete enough to con-

TABLE III. Electrophoretic Fractions of Proteins in Ascitic Fluid (as % of total protein).

Dog No.	Age (mo)	Date	Alb.	α_1 glob.	α_2 glob.	α_3 glob.	β glob.	Fib.	γ glob.
5159	3+		35.4		14.6			22.9	27.7
5160	3+		42.3		17.6	10.3	15.2	10.9	3.7
5158	5		100						
51104	4	2/ 5	42.2	4.9	2.0	16.7	3.9	7.8	22.6
51104	4+	2/15	25.4		7.9			31.7	34.9
52262	Adult	8/ 5	20.3		30.5			30.5	18.7
52262	"	8/14	47.0		18.4	22.0	12.5		
52263	"		28.4	8.5	10.8	10.8	8.0	7.4	26.1

firm the impression but it might be noted that the 8 adult animals in the control group had somewhat lower values for alpha$_2$ globulins than the puppy. Similarly in simply irradiated group the lowest value for alpha$_2$ globulin was found in the sole adult member of that group. Where bleeding was superimposed such a possible relationship was not found.

In another communication we have indicated that inability to produce ascites in the adult dog was apparently circumvented by combining repeated hemorrhage with the internal irradiation procedure(3). Holman has reported that hemorrhage superimposed upon a very high fat diet will result in production of cirrhosis in adult dogs(7). These two sets of observations would seem to have in common only the intermittent hemorrhage as an experimental feature which was superimposed on other forms of trauma. As has been pointed out in both these communications chronic hemorrhage alone, even extending over many years, has never been reported to result in cirrhosis or ascites. A possible interpretation is that some protective material present in the circulation is readily depleted by hemorrhage and not replaced with ease.

One animal, 52262, was studied early during bleeding-irradiation procedure and did not show the characteristic changes in albumin and gamma globulin components. However at a later date ascites had developed and the electrophoretic pattern at that time was in accordance with the other ascites dogs' plasmas.

Summary. 1. A series of determinations of the electrophoretic patterns in plasmas of dogs whose livers have been irradiated following intravenous injection of radioactive colloidal gold are presented. These are compared to a similar control series. The irradiated animals had significantly lower serum albumins and significantly higher gamma globulin components. Some suggestive differences in the effect of age on level of the alpha$_2$ globulin are noted. 2. Examination of ascitic fluid samples obtained from irradiated animals showed that all plasma protein fractions appeared in the ascitic fluid, with albumin, alpha$_2$ globulin, fibrinogen and gamma globulin predominant.

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Excretion of Sulfur During Healing of Experimental Wounds.* (21384)

MARTIN B. WILLIAMSON AND HERBERT J. FROMM.†

From the Department of Biochemistry, School of Medicine and Graduate School, Loyola University, Chicago.

The sulfur amino acids, methionine and cystine, appear to be unique in their ability to accelerate the rate of healing of experimental wounds to the same extent, when present in the diet in equivalent amounts(1-3). Earlier experiments have shown that the healing of wounds is dependent on the availability of the sulfur amino acids, rather than on the total protein nitrogen available in the diet (4,5). Since there is essentially no conversion of cystine to methionine in the organism, it may be considered that cystine is the limiting amino acid in the healing process. The role of methionine in the healing of wounds cannot be discounted, however. The methionine of the tissues has been shown to have at least two important functions in the regeneration of tissue in wounds(6); it is the primary source of cystine required for the synthesis of wound proteins; it is required *per se* as an integral part of the wound protein.

Since the availability of the sulfur amino acids has such a marked effect on the replacement of protein in regenerating wound tissue, it might be expected that the normal equilibria of the reactions involving these amino acids would be disturbed after wounding. This situation should be reflected in an altered metabolism of sulfur during wound tissue regeneration. In this paper, the results of a comparison of the sulfur metabolism, as indicated by sulfur excretion, in normal and wounded rats will be described.

Experimental. In the following experiments, female albino rats weighing 200 ± 20 g were used. The animals were maintained on a basal protein-free diet for 5 days prior to wounding. The basal diet consisted of 83 g sucrose, 10 g lard, 2 g corn oil, and 5 g salt mixture(7). The basal diet also contained a vitamin supplement, whose constituents and proportions have been previously enumerated (2). The animals were offered 8 g of diet per day, which was completely consumed before the next daily feeding period, so that the dietary intake of all the animals was isocaloric. Distilled water was permitted *ad libitum.* After 5 days, the hair on the back of the neck and shoulders was removed and an area of skin 4 cm in diameter was incised down to the fascia. The tissue was then removed with the underlying fascia. Control rats were treated in a similar manner except that no wounds were made. The rats were housed in metabolism cages and urine samples collected every 24 hours after wounding. The samples of urine were stored under toluene in a tightly stoppered bottle at 5°C until the analyses could be performed. The urine samples were analyzed for nitrogen (micro-Kjeldahl) and total sulfur(8,9). After hydrolysis of an aliquot of the urine for 30 minutes in 6 *N* HCl on a boiling water bath, the total sulfate was determined as the barium salt(8). In the experiment in which tracer doses of S^{35} labeled DL-methionine and DL-cystine were administered to the rats, the total S^{35} activity of the urine, as well as the total sulfate activity, isolated as indicated above, was determined. The S^{35} activity was measured using a Tracerlab Autoscalar and a Geiger-

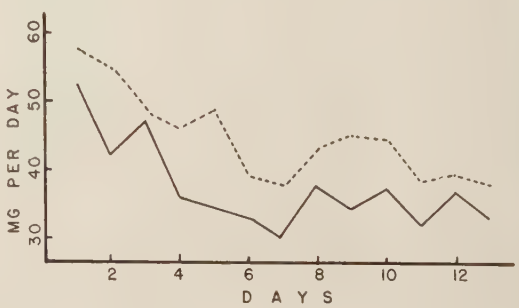

FIG. 1. Excretion of nitrogen by normal and wounded rats plotted in mg/day against days after wounding. Solid line = control rats; broken line = wounded rats.

* This work was done under contract with the U. S. Navy, Office of Naval Research.

† Present address, Dept. of Biochemistry, School of Medicine, Univ. of North Dakota, Grand Forks.

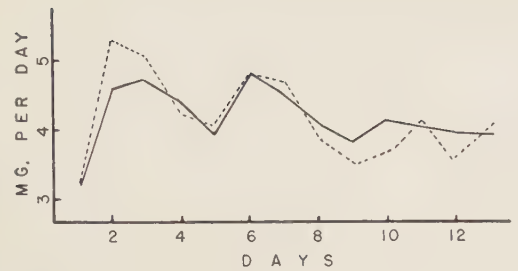

FIG. 2. Excretion of sulfur by normal and wounded rats plotted in mg/day against days after wounding. Solid line = control rats; broken line = wounded rats.

Muller tube having a window thickness of 1.4 mg/cm^2.

Results. Studies on the excretion of nitrogen and sulfur were made in 2 groups of 30 rats kept on a basal protein-free diet. The animals in one group were wounded, while the second group of animals served as controls. The excretion of nitrogen during the first 2 weeks after wounding is shown in Fig. 1. In accordance with the results of previously published experiments(5,10,11), the wounded rats were found to excrete considerably more nitrogen than the control rats. In Fig. 2 is plotted the total sulfur excretion against time after wounding. It can be seen that the wounded and control rats excreted approximately the same amount of sulfur. The data in these two graphs are indicative of the fact that there is an actual retention of sulfur by the wounded rats. It would be expected that the breakdown and metabolism of tissue protein should result in a relatively constant ratio of nitrogen and sulfur excretion, regardless of the amount of nitrogen excreted. However, in this experiment, the ratio of nitrogen to sulfur excreted by the wounded rats was greater than by the controls, indicating a relative net retention of sulfur by the wounded animals.

Fig. 3 shows the excretion of sulfate by the wounded and normal rats. The wounded rats appear to excrete about 40% more sulfate than do the normal control animals. Since sulfate is the end product of sulfur metabolism, this may be taken to mean that the metabolism of the sulfur amino acids is increased in wounded rats as compared to normal ones.

The overall metabolic path of the sulfur in the sulfur-containing amino acids can be abbreviated into the following reactions:

Methionine → Cystine → Sulfate (1).

The importance of cystine during the regeneration of wound tissue led us to study the conversion in the above equation. This was done by following the excretion of S^{35} after the administration of S^{35} labeled methionine and cystine.

The experiment was carried out on 4 groups of 15 rats, maintained on the basal diet. Two groups of rats were wounded as indicated before. Four days after wounding, one group of wounded and one group of unwounded control rats were injected intraperitoneally with DL-methionine-S^{35} (5.90×10^5 counts per minute per rat). The other 2 groups received DL-cystine-S^{35} (6.06×10^5 counts per minute per rat) at the same time. The total S^{35} activity in the urine and the S^{35} activity associated with the sulfate in the urine was determined daily thereafter.

As might be expected from the results in the previous experiment, the total daily excretion of S^{35} was essentially the same for the wounded and control animals. However, the level of S^{35} excretion was higher in those rats receiving the labeled cystine as compared to those receiving the labeled methionine. This is probably due to the fact that the methionine-S^{35} can be utilized either as such or as cystine-S^{35} for protein synthesis and turnover; on the other hand, cystine-S^{35} is utilizable for incorporation into protein only in the unmetabolized state. Thus, there are two forms in which the S^{35} from the methionine-S^{35} can

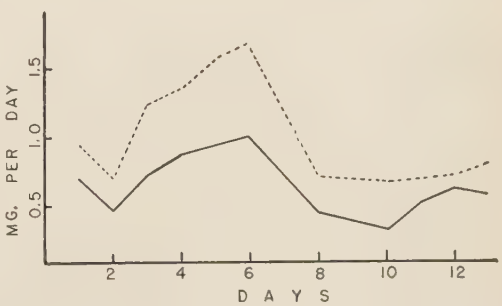

FIG. 3. Excretion of sulfate sulfur by wounded and normal rats plotted in mg/day against days after wounding. Solid line = control rats; broken line = wounded rats.

TABLE I. Excretion of Labeled Sulfate by Wounded and Normal Rats after the Administration of DL-Methionine-S^{35} and DL-Cystine-S^{35}.

Days after wounding	Methionine*		Cystine*	
	Normal	Wounded	Normal	Wounded
5	4680	4840	5300	5300
6	2940	4490	5110	5600
7	2710	4100	4600	7450
8	2290	2500	3900	4500
9	1950	2750	2530	4550
10	1880	2670	2760	4550
11	1785	2230	2630	4430

* Counts/min./24 hr sample, corrected for decay, to time of injection and for difference in dose of methionine-S^{35} and cystine-S^{35}. The labeled amino acids were injected on 4th day after wounding.

be deposited and retained in the tissues, while that from the cystine-S^{35} has only one. This would be expected to result in a greater retention of injected methionine-S^{35} than cystine-S^{35}, and a concomitant greater excretion of injected cystine-S^{35} than methionine-S^{35}.

The data for the excretion of sulfate-S^{35} in this experiment are shown in Table I. It can be seen that the wounded rats which received the methionine-S^{35} excreted more sulfate-S^{35} than did the controls. This must mean that either or both of the series of reactions represented in equation 1) are accelerated in the wounded rats. Data presented in another report(12) indicate that the rate of conversion of methionine to cystine is greater in wounded than in normal rats. In the animals receiving the cystine-S^{35}, it was again observed that the wounded ones excreted more sulfate-S^{35} than did the controls. This can only be interpreted to mean that there is a higher rate of oxidation of cystine in the wounded rats during the regeneration of wound tissue. It may then be concluded that during the process of regeneration of wound tissue, the metabolism of the sulfur amino acids is stimulated.

Summary. The effect of wounding on metabolism of the sulfur amino acids was studied by following the excretion of sulfur in normal and wounded rats. After wounding, there is a relative retention of sulfur, even though a greater amount of nitrogen than normal is excreted. Studies with S^{35} labeled cystine and methionine indicated that the metabolism of these amino acids is greater in wounded than in normal animals.

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Blood and Pituitary Adrenocorticotrophin in Adrenalectomized Rats with Hypothalamic Lesions.* (21385)

S. M. McCann and Katherine L. Sydnor. (Introduced by George Sayers.)

From the University of Pennsylvania School of Medicine, Philadelphia, and Department of Physiology, Western Reserve University School of Medicine, Cleveland, O.

Suitably placed hypothalamic lesions suppress pituitary adrenocortical response to stress as measured by indirect indices of adrenocortical function(1-4). For example, eosinopenia induced by epinephrine and depletion of adrenal ascorbic acid which follows operative trauma do not occur in animals with these lesions(4). Destruction of the median eminence of the tuber cinereum produces these effects in the absence of histologically demonstrable injury to the adenohypophysis(4). In the present experiments the response of rats with hypothalamic lesions has been studied using a more direct indicator of pituitary function, the determination of the concentration of adrenocorticotrophin (ACTH) in blood(5). This method offers two distinct advantages. First, blood ACTH analyses can estimate the rate of increase of ACTH discharge within a few minutes after application of stress. Indirect methods are dependent on changes occurring in the adrenal or in eosinophils one to four hours after application of stress. Second, the method can determine pituitary ACTH output in the adrenalectomized animal, a preparation in which one of the pituitary regulatory factors, namely cortical steroid titer, has been eliminated. Analyses have also been made of pituitary ACTH content, and it is possible to ascribe a decrease in blood ACTH concentration to impaired synthesis or decreased rate of release of the hormone.

Pituitary and blood ACTH have been determined in adrenalectomized rats with hypothalamic lesions which were subjected to the stress of ether anesthesia and bleeding. The data have been compared with pituitary and blood ACTH of rats without such lesions, which were adrenalectomized and given a similar stress. The results indicate that the ACTH content of the pituitary of rats with effective lesions is reduced approximately 50%, whereas blood ACTH concentration is depressed to levels below the detectable range.

Methods. Hypothalamic lesions designed to destroy the median eminence of the tuber cinereum were produced in adult male Wistar rats with the Krieg stereotaxic instrument(4). Three to 8 weeks after the lesion had been placed, the left adrenal was removed under ether anesthesia. One hour later the right adrenal was removed. Adrenal ascorbic acid depletion produced by this severe stimulus was used as a preliminary index of ACTH discharge. Those rats which showed depletion of adrenal ascorbic acid were presumed to have partial lesions of the median eminence. Those which failed to exhibit significant ascorbic acid depletion were classified as rats with effective hypothalamic lesions. Hereafter the term "effective hypothalamic lesions" will be used for lesions which prevented adrenal ascorbic acid depletion. This division of rats into those with effective and those with partial lesions of the median eminence is supported by the histological evidence obtained in previous work(4), which indicated that partial lesions of the median eminence are associated with ascorbic acid depletion in response to unilateral adrenalectomy, while subtotal or total destruction of the median eminence abolishes this depletion. All adrenalectomized rats were maintained on desoxycorticosterone acetate (DCA) by injection in a dose of 0.1 mg per day for 2 weeks. During this time the usual chow ration was supplemented with horsemeat. Fourteen days after adrenalectomy the animals were anesthetized with ether and bled from the abdominal aorta during the third minute of anesthesia. Blood was collected in a heparinized syringe and immediately

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TABLE I. Blood and Pituitary ACTH in Adrenalectomized Rats with Hypothalamic Lesions.

Type of rat	No. of rats	Blood ACTH conc. (mu*/100 ml)	Pituitary ACTH content (mu/gland)	Adrenal ascorbic acid Initial (mg/100 g adrenal)	Adrenal ascorbic acid Depletion (mg/100 g adrenal)	Daily water intake (ml/day)	Wt of both adrenals (mg)
No lesion	16	11 ± 2†	215 ± 27	368 ± 17‡	173 ± 15‡	37 ± 2	35 ± 1
Lesions of rostral hypothalamus	14	18 ± 3	236 ± 44	443 ± 9	167 ± 13	36 ± 2	35 ± 1
Partial lesions:							
Pool #1	10	<4	108 ± 15	470 ± 19	197 ± 19	93 ± 11	27 ± 2
Pool #2	13	5 ± 1.5	—	457 ± 12	207 ± 11	67 ± 8	33 ± 3
Effective lesions:							
Pool A	13	<3	93 ± 17	423 ± 20	1 ± 10	239 ± 15	27 ± 2
Pool B	14	<2	124 ± 16	422 ± 19	−15 ± 9	251 ± 23	28 ± 2
Effective lesions with hypertrophy	11	<2	—	428 ± 24	−33 ± 9	408 ± 21	51 ± 1

* One mu equals one-thousandth of a U.S.P. unit of ACTH. † All values, mean ± stand. error of mean. ‡ Mean computed from 10 rats.

added to 4 volumes of glacial acetic acid. The blood-glacial acetic acid mixtures from a suitable number of rats were pooled and processed by the oxycellulose technic(5). This technic recovers 96 ± 6% of added ACTH activity(6). Assay for ACTH in oxycellulose eluates was performed by the method of Sayers, Sayers, and Woodbury(7). Two or three dose levels of unknown and of standard ACTH were employed. The anterior pituitaries were ground in 0.1 N HCl (0.5 ml per anterior lobe), and the mixture stored in the deep freeze. Pools of 10 or more lobes were assayed for ACTH content in hypophysectomized rats as described above. At autopsy the suprarenal area was examined grossly for adrenal remnants, the testes were weighed, and the brains were placed in 10% formalin. Six brains and 5 pituitaries were serially sectioned to ascertain the location of the hypothalamic lesions and the histological condition of the pituitaries. Daily water intake was measured on several occasions in all rats with lesions.

Results. Previous work(8) has demonstrated that blood ACTH concentration of intact non-stressed rats is less than 0.5 mu ACTH per 100 ml. Two weeks after adrenalectomy the titer has increased to 4 mu per 100 ml. If adrenalectomized rats are anesthetized with ether and bled, the concentration rises to 12 to 16 mu per 100 ml within a few minutes after initiation of anesthesia. All rats in the present study were adrenalectomized two weeks prior to bleeding under ether anesthesia. Except for maintenance on DCA instead of sodium chloride, the procedures were similar to those employed previously.

Controls. Normal rats uniformly exhibited adrenal ascorbic acid depletion from the stress of unilateral adrenalectomy. Two weeks after adrenalectomy, on imposing the stress of etherization and exsanguination, the ACTH concentration was 11 ± 2 mu per 100 ml of blood. Pituitary ACTH content of such rats was 215 ± 27 mu per gland. These figures from DCA-maintained adrenalectomized rats (Table I, line 1) agree with those previously described for adrenalectomized rats maintained on sodium chloride(5,6,8).

Lesions of Rostral Hypothalamus. Rats with lesions placed rostral to the median eminence of the tuber cinereum were similarly studied. These rats did not have diabetes insipidus as judged by daily water intake. This indicates that the lesions did not interrupt a significant fraction of the supra-opticohypophyseal tract. These rats had adrenals of normal size and reacted to stress of unilateral adrenalectomy with depletion of adrenal ascorbic acid. Two weeks following adrenalectomy the animals were anesthetized with ether and bled; blood ACTH concentration in these rats was 18 ± 3 mu per 100 ml and pituitary ACTH content was 236 ± 44 mu per gland, values not significantly different from controls without hypothalamic lesions (Table I).

Partial Lesions of the Median Eminence. Some of the rats with lesions designed to destroy the median eminence of the tuber cinereum exhibited adrenal ascorbic acid depletion in response to operative stress. This was presumably due to failure of the lesion to destroy a sufficient area of this region to block the response. These rats had little or no sign of diabetes insipidus as judged by 24-hour water intake. Despite the fact that ascorbic acid depletion occurred, adrenal weights of many of these animals was less than normal. Blood from the first pool (Pool 1) of rats of this type did not contain a definitely detectable quantity of ACTH. For this reason, in the second pool (Pool 2), the assay was repeated using a larger volume of blood. A level of 5 mu per 100 ml of blood was demonstrated. This concentration is definitely subnormal. The pituitary ACTH content (Pool 1) was 108 $\pm$ 15 mu per gland, which is approximately 50% of normal.

Effective Lesions of the Median Eminence. As stated above, these rats were selected on the basis of absence of significant ascorbic acid depletion in response to unilateral adrenalectomy. Marked variation in adrenal weight was found in this group of rats. Some animals exhibited definite adrenal hypertrophy. It appeared possible that hypertrophy was due to a state of chronic stress. Under these conditions adrenal ascorbic acid depletion might not be a valid index of ACTH secretion. For this reason rats with adrenal weight greater than the mean of normal rats of this colony by more than two standard deviations were arbitrarily placed in a group of rats designated "effective lesions with adrenal hypertrophy." Blood from rats in this category was assayed separately.

Two pools (Pool A and Pool B) of blood and pituitaries were collected from rats with effective lesions, but without adrenal hypertrophy. After processing, each pool was assayed separately in hypophysectomized rats. These rats with effective lesions failed to exhibit ascorbic acid depletion and had on the average subnormal adrenal weights. Testicular atrophy was present in about 35% of cases. All rats with effective lesions had severe diabetes insipidus indicating subtotal or total destruction of the supra-opticohypophyseal tract. Blood ACTH concentration was depressed in these rats to the point where no detectable ACTH was found even when the equivalent of 12 ml of blood was injected into each hypophysectomized rat. Since this assay detects 0.25 mu of ACTH, the ACTH concentration was probably less than 2 mu per 100 ml in rats with effective lesions, a value one-sixth that found in rats without lesions. The pituitaries contained 75 to 124 mu per gland, an amount not significantly different from that found in rats with partial lesions. A separate pool of blood was collected from rats with hypertrophic adrenals and showing no ascorbic acid depletion. This was assayed at a single dose equivalent to 15 ml blood per hypophysectomized rat. No detectable ACTH was found, indicating that the concentration was probably less than 2 mu per 100 ml. Data obtained in this group of rats with effective lesions and adrenal hypertrophy are indistinguishable from those found in rats with effective lesions but without hypertrophic adrenals. Additional data(9) suggest that in the rats with hypertrophic adrenals the extremely severe diabetes insipidus constituted a chronic stress sufficient to produce adrenal hypertrophy even though the acute stress response was completely blocked.

Serial sections of four selected brains from rats with effective lesions have demonstrated that the lesions interrupted the supraopticohypophyseal tract, either in the median eminence or just rostral to this region. The adenohypophyses were histologically normal in the 3 cases examined.

A gain in body weight invariably occurred after placement of hypothalamic lesions. All rats with lesions lost on the average of 3 to 10% of their body weight after adrenalectomy. On the other hand rats without lesions gained about 5% in weight after adrenalectomy. Since rats with lesions of the rostral hypothalamus lost weight after adrenalectomy and had normal values for pituitary and blood ACTH, the changes in body weight appear to be unrelated to the results.

Relation between polydypsia and blood

ACTH concentration. If all groups of rats are considered, it is apparent that lesions which failed to influence water intake were associated with normal blood ACTH concentrations. Those lesions which produced a mild diabetes insipidus were followed by a reduction in ACTH concentration, while those which resulted in maximal diabetes insipidus were associated with blood ACTH levels below the detectable range. An inverse relationship appears to exist between the increase in water intake and the blood ACTH concentration in rats with hypothalamic lesions.

Discussion. In the intact rat acute stress elevates blood ACTH titer from values below the detectable range to 2 mu per 100 ml within 2 minutes of application of the stimulus(8). Adrenalectomy is also followed by an elevation in blood ACTH concentration(8). This is presumably due to removal of the inhibitory effect of circulating adrenal steroids. Blood from the trunk of decapitated adrenalectomized rats contains about 4 mu per 100 ml. This concentration is presumed to represent the titer in non-stressed adrenalectomized rats. Super-imposition of the stress of etherization and exsanguination elevates this titer to 12 to 16 mu per 100 ml within 2 minutes(5,6,8). The effective hypothalamic lesions reported in this study clearly blocked the elevation in ACTH titer observed in stressed adrenalectomized rats and may have blocked the increase which is observed in non-stressed adrenalectomized rats. This suggests that these lesions not only prevent the pituitary response to acute stress, but also block its response to changes in the blood level of cortical steroids. This marked interference with the discharge of pituitary ACTH exists even in the presence of significant though somewhat reduced stores of hypophyseal ACTH. Ganong *et al.*(10), and Fulford and McCann (unpublished) have demonstrated that compensatory adrenal hypertrophy is suppressed by appropriate hypothalamic lesions. This is further evidence that under these conditions the hypophysis no longer responds to a reduction in adrenal steroid concentration by increasing ACTH release.

A significant reduction in blood and pituitary ACTH was demonstrated in rats with partial lesions in the presence of normal values for mean adrenal ascorbic acid depletion from unilateral adrenalectomy. Severe stress of unilateral adrenalectomy may release quantities of ACTH which are greater than that required to produce maximal ascorbic acid depletion. Normal ascorbic acid depletion might still occur, therefore, in rats with partial lesions in which discharge of pituitary ACTH was subnormal.

Adrenal hypertrophy was present in rats with the most severe diabetes insipidus. Control of the diabetes insipidus in such rats(9) returns adrenal weight toward normal. This suggests that in these animals the diabetes insipidus constitutes a chronic stress, which produces a small, sustained increase in ACTH secretion, even in the presence of a lesion which prevents adrenal ascorbic acid depletion and prevents the elevation in ACTH concentration that is found in stressed and non-stressed adrenalectomized rats.

The present study does not elucidate the mechanism by which these lesions block ACTH release. Additional experiments(9) support the concept that this defect is due to interruption of the supraopticohypophyseal tract. This conclusion is based on the significant correlation between severe diabetes insipidus and blockade in ACTH secretion, and on the production of ACTH discharge by large doses of pitressin in rats with effective hypothalamic lesions. In the present study the reduction in blood ACTH appears to be related to the increase in water intake.

Conclusions. 1. Blood ACTH concentration of adrenalectomized rats, treated with DCA for two weeks, and then subjected to the acute stress of ether anesthesia and bleeding was found to be 11 mu per 100 ml. 2. Suitably placed hypothalamic lesions prevented this rise in blood ACTH. 3. Pituitary ACTH was maintained at a level approximately 50% of that found in adrenalectomized rats without lesions. 4. The effective lesions interrupted the supraopticohypophyseal tract as evidenced by their location and by the presence of marked diabetes insipidus.

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Inhibition of Cerebrospinal Fluid Formation by a Carbonic Anhydrase Inhibitor, 2-Acetylamino-1, 3, 4-Thiadiazole-5-Sulfonamide (Diamox).* (21386)

R. D. Tschirgi, R. W. Frost, and J. L. Taylor.

From the Departments of Physiology and Anatomy, University of California at Los Angeles and Veterans Administration Center, Los Angeles.

In view of the reported influence of 2-acetylamino-1,3,4,-thiadiazole-5-sulfonamide (Diamox) on formation of intraocular fluid(1), and the similarity between intraocular and cerebrospinal fluid (CSF) systems, a series of experiments has been undertaken to determine the effect of this compound on CSF formation and intracranial pressure. In every instance, at least a 3-fold reduction in rate of CSF flow, or a decline of approximately 30% in intracranial pressure was observed following intravenous administration of 150 mg/kg soluble Diamox† (acetazoleamide-sodium).

Materials and methods. Cats and rabbits lightly anesthetized with sodium pentobarbital were placed on their sides with heads inclined slightly upwards from the horizontal. To measure CSF flow, a 22-gauge spinal needle was introduced into the cisterna magna and the rate at which drops of CSF appeared was recorded. The volume of each drop was found to be 0.04 cc. The CSF was collected in successive samples for periods of 15 minutes, or until sufficient fluid had accumulated for analysis of sodium, potassium and calcium in the flame spectrophotometer. In other animals, intracisternal pressure was measured by means of a pressure transducer. All animals were artificially respirated and carotid arterial blood pressure was recorded continuously. Tubocurarine was administered throughout each experiment in quantities sufficient to maintain complete inhibition of spontaneous respiration. This procedure was necessary to prevent the large fluctuations in intracranial pressure produced by the respiratory efforts of the animals when inspiring high concentrations of CO_2.

Results. Following an initial equilibration period (not illustrated) the rate of CSF flow or the intracisternal pressure, whichever was being measured, remained quite constant in each animal (Fig. 1). After the intravenous administration of 150 mg/kg Diamox, the rate

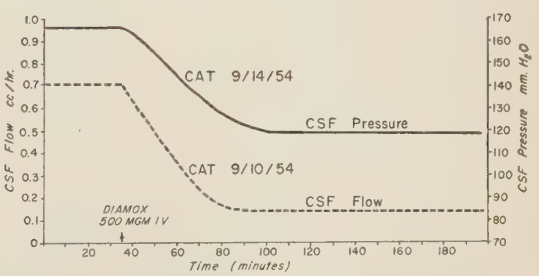

FIG. 1. Representative curves from 2 different cats illustrating effect of intravenous Diamox on cerebrospinal fluid formation and pressure.

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of CSF flow declined over a period of one hour to a new steady rate approximately ⅓ its previous value. In other animals, the post-Diamox flow rates ranged from 1/3 to 1/15 their initial values. Similarly, in a typical experiment the intracisternal pressure fell from 170 mm H_2O to a new level of 120 mm H_2O within one hour. These post-Diamox values remained essentially constant for 2 hours, at which time the experiments were terminated. No significant change in blood pressure was observed throughout the experimental period. Analyses of cat CSF collected before and after the administration of Diamox revealed no significant change in concentration of sodium (154 meq/L), potassium (2.3 meq/L) or calcium (2.6 meq/L). Because of the extremely slow rate of CSF flow following Diamox, however, it is questionable to what extent the samples obtained during this period were representative of newly formed CSF.

In view of the known action of Diamox as a potent inhibitor of carbonic anhydrase, which catalyzes the reaction $CO_2 + H_2O \rightleftharpoons H_2CO_3$, several experiments were performed to investigate the effect on CSF flow and pressure of altering the concentration of inspired CO_2 before and after Diamox administration. After a steady CSF flow rate had been established in a normal animal being ventilated with 100% O_2, changing to a 70% O_2-30% CO_2 mixture produced an immediate increase in CSF flow. However, there was a rapid return to the same steady state flow rate observed during 100% O_2 ventilation, even though the 70% O_2-30% CO_2 inspired mixture was continued for 2 hours (Fig. 2). At the end of this period when respiration was returned to 100% O_2, the flow rate fell precipitously but again rapidly returned to its previous steady state value. Similarly, following Diamox administration, prolonged alteration of the inspired CO_2 concentration produced only transient changes in CSF flow. When, instead of CSF flow rates, intracisternal pressure was measured, similar results were obtained.

Discussion. Diamox has been reported to interfere with blood transport of CO_2 in anesthetized dogs(2). It might, therefore, be reasoned that the inhibition of CSF flow by

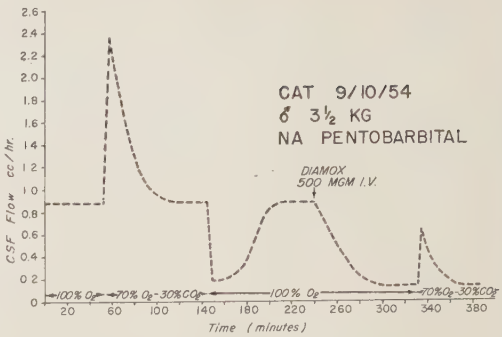

FIG. 2. Representative curve illustrating effect of varying the inspired CO_2 tension on cerebrospinal fluid flow before and after Diamox administration.

Diamox resulted from an increased CO_2 tension of the intracranial fluids. This hypothesis is unlikely since large changes in inspired CO_2 concentrations produced only temporary changes in CSF flow and pressure. These transitory alterations in flow and pressure can best be explained as the result of rapid changes in the volume of the intracranial vascular compartment accompanying cerebral vasomotion in response to changing CO_2 tensions.

As seen in Fig. 2, inhalation of 30% CO_2 following Diamox produces a smaller transient rise in CSF flow than before Diamox administration. The increase in plasma CO_2 tension caused by Diamox itself results in partial dilatation of the cerebral vasculature, and thus, further increases in the CO_2 tension via inhalation of CO_2 mixtures cannot produce as great an increase in vascular volume as before Diamox was administered. This elevated plasma CO_2 tension resulting from Diamox injection can occasionally be observed to produce a temporary rise in CSF flow or pressure immediately preceding the characteristic fall.

In view of the reported occurrence of appreciable amounts of carbonic anhydrase in the central nervous system(3), it is proposed that the effect of Diamox on CSF flow and pressure results from the inhibition of this intrinsic carbonic anhydrase. It is assumed that steady state flow rate as measured in these experiments is representative of the rate of formation of CSF. The following model is, therefore, suggested to account for the movement of water and electrolytes from the plasma into the CSF compartment. Within the central ner-

vous system an appreciable amount of the CO_2 from cellular metabolism does not diffuse into the blood as CO_2, but is rapidly hydrated to carbonic acid in the presence of carbonic anhydrase. This reaction is assumed to occur in cellular elements separating the blood from the extravascular compartments of the central nervous system, *i.e.*, the blood-brain barrier. The H^+ and HCO_3^- ions thus formed are quickly exchanged for other electrolytes, largely sodium and chloride, from the plasma. The view that H^+ and HCO_3^- ions do not rapidly diffuse in any simple fashion between the plasma and the CSF is indicated by the observation that intravenous injection of $NaHCO_3$ solutions markedly increases the alkali reserve of the plasma while changes in cerebrospinal fluid alkali reserve are slight and retarded(4), and the report of Cestan, Sendrail and Lassalle (5) that a considerable acidosis caused by intravenous infusion of HCl produces no decrease in CSF pH. Because of this barrier to free diffusion of electrolytes between the plasma and extravascular fluids of the central nervous system(6) a net increase of two osmols of NaCl in the extravascular fluids will result from each mol of CO_2 thus exchanged. Water, which moves freely among all the intracranial compartments(7) enters from the plasma to reestablish osmotic equilibrium.

This mechanism is hypothesized to exist not only in the choroid plexus, but throughout the entire parenchymal vasculature of the central nervous system, with the exception of the arachnoid villi in the dural sinuses. The subarachnoid CSF is thus considered to be a composite of fluid formed by the choroid plexus moving from the ventricular system into the cisterna magna and thence over the convexity of the brain, and of interstitial fluid flowing outward through the pial surface of the nervous system. This would account for the simultaneous appearance of intravenous radiotracers in the ventricular and subarachnoid fluids when all direct communication between these fluid spaces is blocked(8), and the more rapid equilibration between plasma and intracisternal fluid than between plasma and ventricular fluid following the intravenous injection of D_2O in normal humans(9). The entire net gain of intracranial extravascular electrolytes and water is thus envisioned as moving into the subarachnoid space and back into the blood stream through the arachnoid villi under a hydrostatic pressure gradient, which is determined by, among other factors, the rate of CO_2 production by the central nervous system. The maximum rate of extravascular fluid production predicted by this hypothesis can be calculated for man on the assumption that all the CO_2 produced by the CNS is hydrated and exchanged for NaCl and that the blood-brain barrier is otherwise completely impermeable to NaCl. Since it is highly unlikely that either of these conditions is actually achieved, the calculated results would be expected to be high. Accepting a CO_2 production of 46 ml/min by the human brain(10), 13.5 ml/min of isotonic NaCl could be moved from the plasma into the extravascular compartments. This figure greatly exceeds the generally accepted values for rate of CSF production(11) and the proposed mechanism is therefore theoretically capable of producing the observed water movement.

On the basis of this hypothesis it is possible to account for the decrease in CSF flow and pressure following Diamox administration in the following manner. Carbonic anhydrase inhibition in the central nervous system allows the metabolic CO_2 produced by the cells to diffuse freely into the blood plasma before any appreciable hydration to H^+ and HCO_3^- has occurred. Such rapid equilibration of molecular CO_2 between the plasma and extravascular fluids of the central nervous system is evidenced by the results of Leusen(12) who found that CSF pH paralleled the blood pH changes following inhalation of CO_2 rich gas mixtures. Therefore, after Diamox, the rate of formation of osmotically active particles in the extravascular fluids is considerably diminished, and net water movement is proportionately reduced. Interference by Diamox with a similar mechanism in the kidney tubules is thought to be responsible for the lack of water reabsorption and consequent diuresis produced by this compound(13).

The observation that prolonged shifts in respiratory CO_2 do not produce changes in the steady state rate of CSF formation (Fig. 2) is understandable when one considers that the

increased plasma CO_2 tension will rapidly equilibrate throughout all the intracranial water, resulting in equal changes in pH on both sides of the blood-brain barrier. Thus, no net difference in osmotically active particles between the plasma and interstitial fluid results from this procedure. Rather, a new baseline of CO_2 concentration is established throughout the intracranial contents, and the rate at which CO_2 is produced metabolically by the cells of the central nervous system remains the governing factor in determining the rate of net water movement. After the inhibition of carbonic anhydrase by Diamox, the same explanation suffices for the lack of effect of CO_2 on the steady state rate of CSF production. It is a corollary to this explanation that raising the inspired CO_2 concentration sufficiently to interfere with the metabolism of the central nervous system should result in a diminished rate of CSF flow. This is indeed the case at concentrations above 50% CO_2.

No alteration in CSF sodium concentration would be expected after Diamox administration, all other factors remaining equal, since the reduced rate of net sodium entry into the interstitial fluid resulting from the reduced rate of H^+ formation available to the blood-brain barrier is isotonically balanced by a diminished net water movement.

Summary. Intravenous administration of a carbonic anhydrase inhibitor, 2-acetylamino-1,3, 4-thiadiazole-5-sulfonamide sodium (Diamox), to anesthetized cats and rabbits caused a sustained 3- to 15-fold decrease in rate of cerebrospinal fluid formation resulting in a marked reduction in intracranial pressure. This phenomenon cannot be ascribed to changes in plasma CO_2 concentration since prolonged alterations in alveolar CO_2 had no effect on the steady state rate of CSF production either before or after Diamox. It is hypothesized that normally the carbonic anhydrase of the central nervous system (CNS) accelerates the formation within the blood-brain barrier of H^+ and HCO_3^- from CNS CO_2. These ions are exchanged for plasma anions and cations, largely Na^+ and Cl^-, which thus enter the interstitial fluid of the CNS. By virtue of the relative impermeability of the blood-brain barrier to electrolytes, water enters from the plasma to maintain osmotic equilibrium. This mechanism is considered to exist not only in the choroid plexus but throughout the CNS parenchymal vasculature. The interstitial fluid thus formed flows through the pial surface of the CNS into the subarachnoid space and returns to the blood through the arachnoid villi. By decreasing the rate of formation of H^+ and HCO_3^- within the blood-brain barrier, Diamox diminishes the rate of formation of CNS interstitial fluid and cerebrospinal fluid.

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Reversal of Ergotoxine Inhibition of Deciduoma by M.E.D. of Progesterone in Spayed Pseudopregnant Rats. (21387)

M. C. Shelesnyak.

From the Department of Experimental Biology, Weizmann Institute of Science, Rehovoth, Israel.

It has been demonstrated that the subcutaneous injection of a single, small dose of ergotoxine ethanesulphonate inhibits deciduomata formation in the rat, and that this inhibition can be reversed by the administration of progesterone(1). Similar treatment with ergotoxine also disturbs the normal estrus cycle, terminates pseudo-pregnancy and early (1st trimester) pregnancy in the rat(2). It was postulated that the ergotoxine upset the hormonal balance of the rat by acting on the pituitary via the hypothalamus(2). A series of experiments was set up to test this postulate. This is a report of an investigation designed to ascertain whether a target organ competition or a direct antagonism exists between ergotoxine and progesterone.

Methods. Albino rats weighing between 160-320 g were used. Series I. 50 females (160-200 g) were separated into 10 equal groups. After establishing the regularity of the estrous cycle, pseudopregnancy was induced in all by electrical stimulation(3). On the fourth day of pseudopregnancy the antemesometrial wall of one horn of the uterus was traumatized by scratching. Four of the 10 groups were bilaterally ovariectomized at the same time; the ovaries remained intact in the other 6. Various combinations of ergotoxine ethanesulphonate (in ethanol) and progesterone (in sesame oil) were injected at the time of the operations; ergotoxine, once, and progesterone daily for 3 days in all groups but one, (Table I). On the day following the third progesterone injection, the animals were sacrificed and the uteri examined. Fixation in neutral (4%) formalin and staining with Harris haematoxylin and eosin was done for microscopic study.

Results revealed that decidual reactions, ranging from microscopic nests to small macroscopic growths, developed in all groups of which the animals received M.E.D. of progesterone established by Astwood(4) as required for maintaining the spayed pseudopregnant uterus in condition to respond to trauma to develop deciduoma. When larger quantities of progesterone were used, the decidual reaction was increased, but very large growths were rare. This response occurred whether the animals received ergotoxine or not; and whether their ovaries were intact or removed. (Table I).

Series II. 30 rats (240-320 g), 15 ♂♂ and 15 ♀♀ were used to test whether there existed a direct ergotoxine-progesterone antagonism. Twenty animals (10 ♂♂ and 10 ♀♀) received 100 mg of progesterone 24 hours prior to a second injection of 100 mg of progesterone which was followed, immediately in 10 cases, or 1 hour later in 10 cases, by intraperitoneal injection of ergotoxine (100 mg/kg body weight). The remaining 10 animals which received only ergotoxine served as controls.

Results. In no case did the progesterone protect the animals from the severe toxic to lethal effects of the ergot alkaloid.

Discussion. Considering the chain of events beginning with a neurochemical stimulation of the pituitary by the hypothalamus → hormone (gonadotrophin) release → follicular and lutein secretions by the ovary → preparation of a progravid type endometrium → deciduoma formation following uterine trauma, it could be possible for the ergotoxine action to occur at any of many points. The capacity for progesterone, administered at the same time as the ergotoxine, to reverse the ergotoxine inhibition of deciduoma formation, could reflect a direct ergotoxine-progesterone antagonism, or a target organ (uterus) competition. If either of these mechanisms is the operative one, then a quantitatively scaled relationship should exist between the amount of ergotoxine administered to suppress decidual growth and the amount of progesterone needed to reverse or override the suppression. The fact that in conditions, both with and without intact

TABLE I. Ergotoxine Inhibition of Deciduoma and Its Reversal by Progesterone in Normal and Spayed Pseudopregnant Rats. Ten groups, 5 cases each.

	Ovary intact						Ovary removed			
Ergotoxine (mg/inj.)	.5	1.0	.5	.5	1.0	3.0	.0	.5	1.0	3.0
Progesterone (mg/inj. × No. days)	.0	.5×1	.15×3	.25×3	.25×3	.25×3	.25×3	.25×3	.25×3	.25×3
Decidual reaction	—	—	±→+	+	+	+	+	+	+	+

ovaries, the M.E.D. for induction and maintenance of deciduoma (Astwood) was sufficient, even with quantities of ergotoxine 12 times the M.E.D. for deciduoma inhibition, rules out the existence of target organ competition between ergotoxine and progesterone, and a direct antagonism between the drug and the hormone. The failure to protect animals from lethal doses of ergotoxine with very large quantities of progesterone confirms the nonexistence of direct antagonism.

It is evident from these experiments that the site of action or ergotoxine in inhibiting deciduoma formation is at a level higher than progesterone antagonism or endometrial competition.

Summary. Minimum effective dose of progesterone for deciduoma growth in the spayed pseudopregnant rat was effective in reversing ergotoxine inhibition of deciduoma, even when 12 times as much ergotoxine as necessary was used. Progesterone did not protect rats against toxic effects of ergotoxine.

The author is greatly indebted to Mr. Shalom Joseph for his valuable technical assistance.

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Protein Breakdown in Thermal Injury.* (21388)

GEORGES UNGAR AND EVELYN DAMGAARD.

From the Institution for Tuberculosis Research and Department of Physiology, University of Illinois College of Medicine, Chicago.

Increase in proteolytic activity has been shown to occur in thermal injury by several groups of workers(1-5). Experimental results presented in this paper show an increase of protein breakdown in tissue slices submitted to heat and suggest that this proteolysis is caused, at least partly, by the activation of an enzyme system present in normal tissue as an inactive precursor.

Methods. Most experiments were performed with guinea pig skin. Albino guinea pigs were killed by bleeding, the previously shaved abdominal skin was removed, freed from subcutaneous tissue and cut into slices of 2 to 3 mm with fine scissors. Samples of 1 g of skin were suspended in 20 ml of buffered saline (pH 7.4 phosphate buffer diluted to 0.15 M with 0.85% NaCl). The buffer solution was heated to the requisite temperature before addition of the skin slices. The skin suspensions were heated for 5 minutes and after this interval placed in a 37.5°C water bath for a further 20 minutes. A control sample, run with each series of heated samples, was kept at 37.5°C throughout the experiment. Aliquots of 1 ml were taken from each sample at the end of the 5-minute heating period and at 5-minute intervals thereafter. The aliquots were mixed with 4 ml of 15% trichloracetic acid and, after one hour standing, filtered on Whatman No. 50 filter paper. The

* These studies were aided by a contract between the Office of Naval Research and the University of Illinois.

filtrates were read in the Beckman spectrophotometer at 280 mμ against a blank containing the same concentration of trichloracetic acid or any other reagent added to the samples. In most experiments, pooled skin slices, taken from 3 to 4 guinea pigs, were used. Experiments were performed with rat skin using the same procedure with 500 mg samples instead of 1 g. The increase in optical density readings observed in the course of the experiments was interpreted as due to the gradual breakdown of tissue proteins and, accordingly, the results are expressed in terms of mg of protein hydrolyzed per g of tissue. These values were obtained, after correction for the volume of suspension fluid, by dividing the optical density readings with 0.9 which was the mean extinction coefficient of the saline extractable skin proteins. This conversion, although certainly an approximation, indicates the correct order of magnitude of the protein breakdown. When enzyme inhibitors were used, these were dissolved in the suspending medium, before addition of the skin slices. After several unsuccessful attempts at extracting a protease from skin with saline, lung tissue was used. Guinea pig lungs were cut into slices and submitted to various temperatures according to the procedure described for skin. Immediately after heating, the slices were ground up in a Waring blender kept at low temperature. The homogenate obtained was centrifuged at 5000 r.p.m. for 20 minutes. The supernatant was diluted 20-fold with distilled water and the pH adjusted to 5.2 with acetic acid. The resulting globulin precipitate was collected by centrifugation and redissolved in buffered saline. Amounts of this solution corresponding to 200 mg of tissue were tested for protease activity with the method previously used for fibrinolysin estimation and described in detail elsewhere(6,7). The method uses fibrinogen as a substrate and the amount hydrolyzed is estimated by the ultraviolet absorption of the non-clottable fraction.

Results. Variation of proteolysis with temperature. Fig. 1 shows the results of a representative experiment in which skin slices were heated to 50, 60, and 90°C. It is seen that

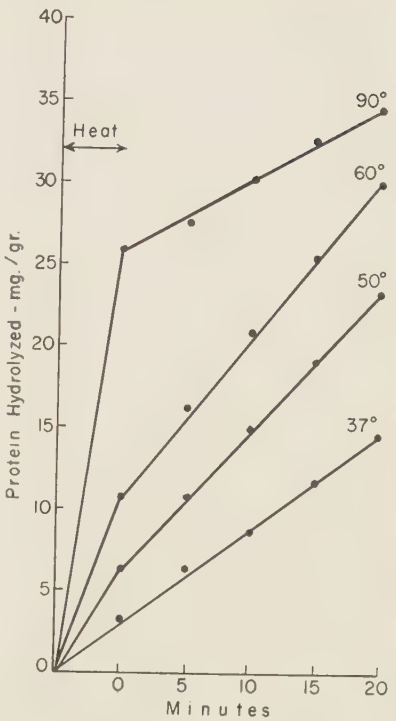

FIG. 1. Protein breakdown in heated skin slices. At 0 min., after 5 min. exposure to the specified temperatures, the tissue suspensions were kept at 37.5°C for 20 min.

proteolysis proceeds at a faster rate during heating than during the following 20 minutes. In evaluating the action of heat on protein breakdown it seemed therefore necessary to distinguish 2 phases: an initial and a secondary phase. The extent of the phenomenon was expressed in terms of proteolysis rate: mg of protein hydrolyzed per g of tissue per minute. The results are also expressed in terms of the relative rate. This was obtained by dividing (in each series of experiments) the rate of the heated samples by the rate of the control in that particular set of experiments. This treatment seemed justified in view of the variations observed in the control rate (range 0.33-0.66). The results indicate that heating increases the rate of initial proteolysis throughout the range studied (37.5-96.5°C), while secondary proteolysis increases only up to about 60°C, after which it falls, some time even below the control level.

Fig. 2 shows the relative rate of initial

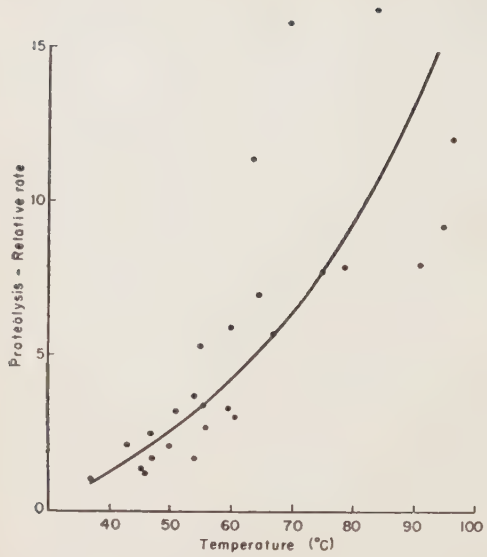

FIG. 2. Relative rate of initial proteolysis as a function of temperature. Skin slices were exposed to heat for 5 min.

proteolysis as a function of temperature. The function is probably not linear and the best fitting line was drawn by inspection. The correlation coefficient ($r = 0.75 \pm 0.17$ standard deviation) was found highly significant.

Fig. 3 illustrates the variation of secondary proteolysis with temperature. The best fitting lines were calculated by means of the least squares method; the ascending line with points between 37.5 and 65.0°C and the descending line with those between 67.0 and 83.5°C. The correlation coefficient for the points up to 65°C ($r = 0.79 \pm 0.07$, standard deviation) was found highly significant.

Similar results were obtained with rat skin. Skin slices from 15 rats were heated to temperatures between 58 and 62°C; each had a control sample kept at 37.5°C. The initial rate for the controls was 0.56 (± 0.10) and for the heated skin 2.50 (± 0.98); the secondary rate was for the controls 0.54 (± 0.09) and for the heated samples 0.78 (± 0.21). The differences were highly significant with the t test. The relative rates were 4.45 and 1.40, respectively.

Inhibition of proteolysis. To investigate the enzymatic nature of the protein breakdown induced by heat, protease inhibitors were added to the suspending medium of the skin slices and proteolysis rate was estimated as described above. Soy bean trypsin inhibitor† and sodium salicylate(8) were used in most experiments and it is seen in Table I that both compounds inhibited the secondary proteolysis at all temperatures tested, even at the control level of 37.5°C. The initial proteolysis, however, was inhibited only at low temperatures. Inhibition decreased with higher temperatures and in the 60 to 100°C range no inhibition was observed. It was concluded that part of the initial proteolysis was produced by a mechanism similar to that of the secondary breakdown but the major component was caused by a different process. The enzymatic nature of the initial proteolysis is unlikely since such strong inhibitors as $CuSO_4$ and $HgCl_2$ failed to inhibit it. An attempt was made to see whether the simple physical action of heat can cause a breakdown of proteins. A number of 0.5% solutions of proteins (whole serum protein, serum albumin, fibrinogen, hemoglobin, egg albumin, casein) were submitted for 5 minutes to 95°C but no increase in protein breakdown products could be detected in the trichloracetic filtrate.

Presence of a protease in heated tissue slices. When suspensions of skin slices were

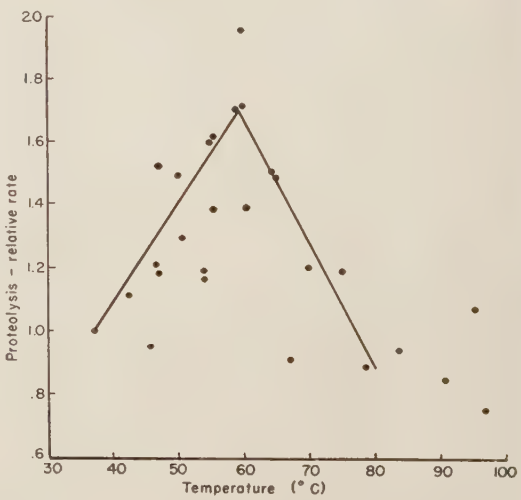

FIG. 3. Relative rate of secondary proteolysis as a function of temperature. The best fitting lines were calculated with the least squares method.

† A crystalline protein obtained from General Biochemicals, Chagrin Falls, O.

TABLE I. Action of Protease Inhibitors on the Rate of Heat-Induced Proteolysis in Guinea-Pig Skin Slices.

Inhibitor	Temp., °C	Rate of proteolysis* Initial: No inhibitor	Initial: With inhibitor	Secondary: No inhibitor	Secondary: With inhibitor
SBTI† .5 mg/ml	37.5	.42	.25	.41	.30
" .5	51.5	.92	.58	.58	.37
" .1	58.0	1.17	.58	.46	.33
" 2.0	58.0	1.17	.67	.46	.12
" .5	60.5	2.10	1.75	.74	.33
" .5	86.0	5.33	4.75	.44	.40
Na salicylate .005 M	37.5	.47	.27	.45	.27
Idem	46.5	.50	.25	.46	.27
"	54.0	2.16	.50	.67	.39
"	55.0	2.25	1.17	.67	.27
"	65.0	2.92	2.50	.56	.35
"	78.5	4.50	5.00	.50	.25
"	96.5	5.00	4.75	.31	.36
$CuSO_4$ .01 M	37.5	.55	.00	.55	.00
"	96.5	5.00	5.00	.31	.08
$HgCl_2$ .01 M	96.5	5.00	5.25	.31	.10

* Absolute rates. † Soy bean trypsin inhibitor.

filtered immediately after heating and the filtrate was incubated for 20 minutes, little or no proteolysis was observed. Even when a suitable substrate (fibrinogen) was added to the filtrate no protease activity could be detected. This indicated that the protein breakdown induced by heat takes place in the tissue and that the enzyme responsible for it does not diffuse out into the suspending medium. There is, however, some evidence that *in vivo* the tissue protease does reach the blood stream since it was found in serum(1) and in lymph(5). A protease active on fibrinogen at pH 7.4 was also found in the urine(9-11) of guinea pigs partially immersed into hot water (65°C for 30 sec.). The urinary protease appeared about 30 minutes after burn and was still present 24 hours later.

Attempts were made to extract the protease from heated skin slices with buffered saline. These attempts were unsuccessful; skin protease can, however, be extracted by using higher salt concentrations(3,4) but these may also activate the enzyme precursor.

Since in previous experiments it was found possible to extract a protease from lung tissue with saline(7), lung was used instead of skin for the extraction of enzyme. Lung slices were suspended in buffered saline heated to 43, 50, 56, 60, 64, and 80°C, together with a control kept at 37.5°C. After heating, the tissue slices were extracted and the extract tested for protease activity, using the methods described above. Fig. 4 shows that protease activity of the lung extracts increases with temperature up to 56°C and then decreases abruptly, falling to control levels. It should be noted that low temperatures also activate the tissue protease. The action of cold will be the object of separate studies. It is probable that skin is less sensitive to slight thermal stimuli than lung since it is normally exposed to a wider range of temperatures.

In other experiments, lung slices kept at 37.5°C were extracted and the extract heated to temperatures between 50 and 60°C. No protease activity was detected in these preparations. It is known, however, that the lung extract contains an enzyme precursor(7). It seems, therefore, that certain tissues contain the precursor of a protease which can be activated by heat or cold. The proenzyme itself, however, is not sensitive to thermal stimulation; the activation takes place by the mediation of another tissue constituent which is either destroyed in the extraction procedure or left behind in the centrifugation residue.

Discussion. Increase in proteolytic activity has been detected by various methods: protease activity in serum(1) or lymph(5) of

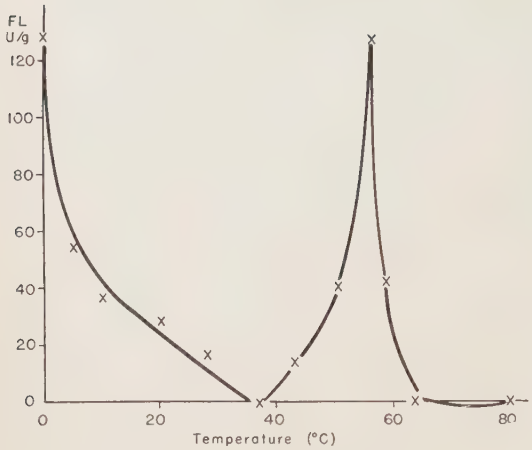

FIG. 4. Protease activity of extracts from heated lung tissue. Abscissa: temperature to which lung slices were exposed before extraction. Ordinate: fibrinolytic units per g of lung. For definition of unit see (6,7).

burnt animals or extraction from burnt tissues (3,4). These results were interpreted by Peters and his coworkers as indicating the release of a protease from the zone of incomplete destruction(2).

The results just reported suggest that the thermal stimulus acts, not by releasing a preformed enzyme, but by converting a precursor into an active protease. A similar activation has been shown to take place in the course of the allergic reaction(6,7,11). Such a process is reminiscent of the blood proteolytic system in which a precursor (profibrinolysin, plasminogen) is converted into a protease (fibrinolysin, plasmin) by various stimuli. The enzyme described in the present report, however, is not fibrinolysin since it has no action on the synthetic substrate *p*-toluyl-sulfonyl-arginine-methylester which was shown to be attacked by fibrinolysin(12). It is more likely to be similar to the enzyme described by Beloff and Peters under the name of dermoproteinase(3) which was shown to be localized almost entirely in the epidermis(13).

The nature of the initial proteolysis induced by heat is at present unknown. In view of its resistance to high temperatures and to the most drastic enzyme inhibitors, it is unlikely to be enzymatic. Axelrod and Martin found that heating a suspension of defatted, saline extracted skin powder to high temperatures increased markedly the amount of trichloracetic soluble nitrogen(14). This, however, as it was shown above, does not occur in protein solutions.

It has been assumed that activation of a proteolytic enzyme system in blood and tissues is an important factor in the mechanism of inflammation and shock(11,15). It is therefore possible that the protein breakdown observed in skin—by liberating histamine and other active substances—plays a role in producing the inflammatory changes associated with the thermal injury and perhaps also some of its systemic manifestations. Rosenthal observed a good correlation between histamine release from the skin and protein breakdown(16). In experiments conducted simultaneously with those reported in this paper(17) it was observed that protease inhibitors injected into animals reduced the inflammatory response to burn injury.

Summary. Protein breakdown was observed when skin slices were submitted to heat *in vitro*. Part of the proteolysis is caused by enzymatic action and the protease responsible for it can be extracted from heated tissue. The results suggest that thermal stimulation acts by converting an inactive enzyme precursor, present in normal tissue, into an active protease. The process described may play a role in the pathogenesis of thermal injury.

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Inhibition of Inflammatory Response in Beta-Irradiated Skin.* (21389)

GEORGES UNGAR AND EVELYN DAMGAARD.

From the Institution for Tuberculosis Research and Department of Physiology, University of Illinois College of Medicine, Chicago.

Ionizing radiations, especially X-rays and beta rays, have been used for many years for the local treatment of inflammatory lesions. The present paper reports experimental results which indicate that rat skin subjected to beta rays does not respond to stimuli which in normal skin elicit an inflammatory response.

Methods. The animals were irradiated by means of a beta ray source of Sr^{90} emitting 6000 rep. per hour, measured at the point of contact with the skin. The diameter of the opening was 25 mm.† Albino rats, weighing 100 to 150 g, anesthetized with Nembutal (30 mg/kg intraperitoneal), were used and the beta ray source was applied to the abdominal skin previously shaved. Some of the irradiated rats were observed for a month, others were tested at specified intervals, as described below. All of the 22 rats irradiated with 3000 rep. (30 min. application of the source) and kept for a month exhibited definite skin injuries at the site of irradiation. These started between the 6th and 10th days with a circular erythema followed by desquamation and sloughing. The peak of the reaction was reached around the 15th day and was followed by slow healing. On the 30th day a discolored scar, surrounded by a zone of depilation, marked the irradiated spot. No systemic effects were noted at any time. According to Zirkle(1), in adult rats, beta rays are almost entirely absorbed by the skin. Inflammatory response was produced in the skin either by heat or by chemical means. Heat was applied by a copper plate (diameter 18 mm.) attached to a glass cylinder in which hot water was flowing. As a routine, temperature of 65°C was applied for 30 sec. Skin response was also elicited by intradermal injections of 0.1 ml of saline in which 0.1 mg of histamine dihydrochloride or 0.1 mg of the "histamine liberator," compound 48-80(2)‡ was dissolved. These comparatively high concentrations proved necessary for obtaining an unequivocal skin response, owing to the relative insensitivity of rats to histamine. The response to both agents consisted in a circular area of swelling surrounded by a diffuse zone of erythema. The edema persisted for about 2 hours, while the erythema disappeared in 20 to 30 min. No consistent grading of the reaction could be achieved; only positive and negative reactions were recorded. Histamine content of the skin was estimated according to the technic described by Feldberg and Talesnik(3): grinding of tissue in mortar, extraction with concentrated HCl and assay of the neutralized extract on isolated guinea-pig ileum. Protein breakdown in skin slices was measured by means of a method previously described(4,5) using as a criterion of proteolysis the ultraviolet absorption of the trichloracetic filtrate.

Results. A marked difference was observed when normal and irradiated skin areas were

* These studies were aided by a contract between the Atomic Energy Commission and the University of Illinois.

† Made to specifications by Nuclear Instruments and Chemical Corp., Chicago, Ill.

‡ Condensation product of N-methylhomoanisylamine with formaldehyde, resulting in the formation of a mixture of di-, tri- and tetramers; kindly supplied by Dr. E. J. deBeer, Wellcome Research Laboratories, Tuckahoe, N. Y.

TABLE I. Influence of Beta Irradiation on Skin Response to Histamine and 48-80, Protein Breakdown *In Vitro* and Histamine Content of Rat Skin.

Dose (rep.)	Interval	Reaction to histamine*	Reaction to 48-80*	Relative rate of proteolysis† Normal	Relative rate of proteolysis† Irradiated	Skin histamine, μg/g Normal	Skin histamine, μg/g Irradiated
3000	1 hr		+ + +	1.45	1.43		
	3	+ + +	+ + —	1.28	.91		
	6		— — —	1.45	.78		
	18		— — —	1.57	.76		
	1 day	+ + +	— — —	1.16	.49	38.5	40.0
	2		— — —	1.50	.32		
	3	+ + +	— — —	1.25	.57	32.0	33.0
	7		— — —	1.43	.86		
	9	+ + +	— — —	1.40	.90	24.5	29.0
	14		+ + +	1.42	1.08		
	17		+ + +	1.80	1.29	32.5	40.5
	24	+ + +	+ + +	1.45	1.32		
	30		+ + +	1.50	1.55		
2000	2 days		— — —	1.40	.85		
	6		— — —	1.36	.76		
	9		+ + +	1.50	1.31		
1000	2 days		+ + —	1.45	1.01		
	6 days		+ + +	1.25	1.14		
	9		+ + +	1.50	1.50		
500	3 days		+ + —	1.40	.96		
	7		+ + +	1.20	1.17		
	9		+ + +	1.18	1.15		

* Only reaction of irradiated skin is tabulated; in normal skin the reaction was always positive. Each + or — sign designates skin reaction of one animal. Groups of 3 rats were used for each time interval.

† Only the "secondary" proteolysis rate was recorded; the initial proteolysis was disregarded. For explanation see (5).

burnt in rats: while normal skin always responded with a distinct edema, developing within the first two hours, irradiated skin showed little or no swelling. No quantitative studies were made with this method but the observations clearly indicated that the irradiated skin lost its ability to respond to the thermal stimulus. Experiments were designed to investigate the mechanism by which this change of responsiveness is brought about.

The first possibility was that beta rays altered the vascular and other tissue structures which react to inflammatory stimuli. Since these structures are known to respond to histamine, intradermal injections of histamine were given to groups of 3 rats at intervals varying from 3 hours to 24 days after irradiation. Injections into both normal and irradiated skin sites always elicited a positive response (Table I).

The next step was to study the action of endogenously liberated histamine. It is seen in Table I that 6 hours after irradiation with 3000 rep., compound 48-80, a "histamine liberator", failed to elicit a response in the irradiated skin area. The unresponsive state persisted at least 9 days. Positive reaction was observed on the 14th day. At the non-irradiated sites the reaction to 48-80 remained positive throughout the period of observation.

In animals irradiated with 2000 rep. the skin reaction of 48-80 was negative for 6 days. With smaller doses of beta rays, 1000 and 500 rep., no definite inhibition of the skin reaction was observed.

A possible explanation of these results was that beta irradiation caused a histamine depletion of the skin and no histamine was left to be released by 48-80. To test this possibility, the histamine content of normal and irradiated rat skin was determined. Skin samples were pooled from groups of 3 rats, 1, 3, 9 and 17 days after irradiation with 3000 rep. It is seen in Table I that beta irradiation does not cause histamine depletion of the skin. There was even slightly more histamine in

irradiated than in normal skin but the difference is within the limit of experimental error. One possibility remained to be explored; that inhibition of inflammation by beta rays was due to interference with the mechanism by which histamine and other inflammation-promoting substances are released. This mechanism has been studied in allergy(4,6) and thermal injury(5). A significant reaction in it is the activation of a protease system causing protein breakdown in tissues.

Systematic experiments were conducted to measure protein breakdown in slices of irradiated and normal skin, according to the technic mentioned above. Groups of 3 rats were killed at varying intervals after irradiation and pools of normal and irradiated skin were weighed into 500 mg samples suspended in 10 ml buffered saline. Sample A was normal skin kept at 37.5°C, sample B was normal skin heated for 5 min. at 60°C and sample C was irradiated skin submitted to the same heat. After heating, all samples were kept at 37.5°C for 20 min. and protein breakdown was followed by aliquots taken at 5 min. intervals.

Results of these experiments are shown in Table I. They are expressed in terms of relative proteolysis rates, obtained for the normal skin by dividing the rate of B by that of A and for the irradiated skin by dividing C by A. It is seen in Table I that heating normal skin to an average temperature of 60°C (range 58-62) for 5 min. increased proteolysis by 40% (± 3, standard deviation). In the beta ray treated skin, however, the proteolytic process was inhibited. The onset and duration of this inhibition depended on the dose of radiation received.

There was a striking parallelism between the inhibition of proteolysis and the skin response to 48-80. The latter tended to become negative whenever the relative proteolysis rate fell below 1.0.

The inhibiting action of beta rays on proteolysis is by no means specific for thermal stimuli. Activation of the proteolytic system of skin was also induced by adding to a suspension of skin slices either compound 48-80 or the bacterial product streptokinase.§ In 6 experiments, 48-80 (0.5 mg/ml) was added to 500 mg of normal rat skin suspended in 10 ml buffered saline and incubated at 37.5°C for 20 min. The relative rate of proteolysis was 1.45 ± 0.04. When streptokinase (10,000 units/ml) was added under similar conditions, the rate was 1.48 ± 0.05. In 5 experiments the same agents were added to beta-irradiated rat skin (3 days after irradiation with 3000 rep.), the relative proteolysis was 0.82 ± 0.04 with 48-80 and 0.76 ± 0.03 with streptokinase. Five control samples of irradiated skin showed a proteolysis rat of 0.46 ± 0.02. These last results indicate that irradiation also depresses the proteolytic reaction elicited by the slicing and handling of the tissue. It seems, therefore, that beta irradiation depresses the ability of the skin to respond to a variety of stimuli by the normal reaction of protease activation.

Discussion. The results just summarized suggest that beta radiation depresses the proteolytic enzyme system which plays a role in the mechanism of inflammation(4-7). Whether other types of radiations share this action of beta rays is not known but Becker observed inhibition of the Shwartzman phenomenon by X-rays(8). On the other hand, X-rays are believed to activate a proteolytic system(9) and cause histamine release(10). Beta radiation does not seem to release histamine from dog skin *in vitro*(11). The nature of the protease system involved in the action of beta rays is discussed elsewhere(4,5). Its sensitivity to radiations is not known but most known proteases are destroyed by ionizing radiations *in vitro*(12).

Recent work has indicated that mast cells are the main source of histamine in the tissues (13). There is also indication that mast cells undergo degenerative changes under the influence of radiations(14). It is not known whether this damage to mast cells can impair the histamine-releasing mechanism and whether mast cells contain other inflammation-promoting substances besides histamine. Histamine release is only part of a fundamental tissue reaction for which it serves as an indicator owing to the fact that histamine is com-

§ Preparation of streptokinase and streptodornase (Varidase), Lederle Laboratories, Pearl River, N. Y.

paratively easy to detect.

The findings reported in this paper may be applied to the early detection of radiation injury. Skin lesions produced by beta rays become visible only about 8 days after irradiation. Skin response of an irradiated area to 48-80 becomes negative between 3 and 6 hours. Compound 48-80 has been used in man and human skin reactions to it are well standardized(15). By means of this method, it may therefore be possible to determine whether a given skin area has been subjected to a damaging dose of radiation.

Summary. Treatment of rat skin with beta rays depresses the local reaction to certain inflammatory stimuli. Response to histamine is not inhibited but the histamine liberator 48-80 fails to elicit a response within a few hours after irradiation. Histamine content of the skin is not diminished after beta irradiation. The results shown above suggest that beta rays act by interfering with a tissue protease system which plays an important role in the mechanism of inflammation. The inflammation-inhibiting action of beta rays may be applied to the early detection of radiation injury.

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Cytolytic Effects of Mumps Virus in Tissue Cultures of Epithelial Cells.* (21390)

GERTRUDE HENLE, FRIEDRICH DEINHARDT,† AND ANTHONY GIRARDI.
(Introduced by Werner Henle.)

From the Children's Hospital of Philadelphia and Division of Virology, Department of Public Health and Preventive Medicine, School of Medicine, University of Pennsylvania, Philadelphia.

Attempts have been made to propagate a number of strains of mumps virus in various stages of chick embryo adaptation in tissue cultures of human cancer cells, strain HeLa, and of monkey kidney epithelium. Although evidence of propagation has been obtained thus far only with recently isolated mumps viruses, characteristic lesions were observed in both types of cells upon first transfer of large amounts of all strains tested. The latter observations form the basis of this report.

Methods and materials. Virus. The Habel, Enders, Barnes, Tucker and D. D. strains of mumps virus were employed. The first two were originally isolated in monkeys and had undergone thereafter numerous passages in

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† On study leave from the Bernard Nocht Institut fuer Schiffs-und Tropenkrankheiten, Hamburg, Germany.

chick embryos, initially by the yolk sac and/or amniotic, and finally by the allantoic routes. The 8th and 46th allantoic passages were used, respectively. The other three strains were isolated directly in chick embryos in this laboratory. The Barnes strain had been passed 9 times amniotically and 8 times allantoically; the Tucker and D. D. strains were in their 2nd to 4th amniotic passages. In the earlier experiments the strains were used after storage at –70°C for periods from 10 months to 3 years. In later tests fresh allantoic or amniotic passages were made by the technics described(1). These were used either immediately or after storage at –20 or –70°C for periods up to 4 months. The fresh preparations revealed infectivity titers in chick embryos in excess of 10^7 ID_{50}/ml and the hemagglutinin concentrations ranged from $10^{3.1}$ to $10^{3.8}$ units/ml. For inactivation by ultraviolet light infected allantoic fluids were dialyzed against phosphate-buffered saline solution of pH 7.0 and irradiated as described elsewhere(2). In most of the experiments reported, allantoically-adapted strains (Barnes and Enders) were employed. *Tissue culture technics.* The strain of HeLa cells(3) was kindly supplied by W. F. Scherer. The cells were grown in bottles in the presence of 40% human serum, 56% Scherer's maintenance solution (M.S.)(4), and 4% acetone-extracted chick embryo suspension. The sheets of cells obtained after 5 to 7 days of incubation were washed 3 times with Hanks' solution and then scraped into a growth medium consisting of 20% horse serum, 60% M. S., 20% "199" and containing 100 units of penicillin and 100 μg of streptomycin per ml. The cells were dispersed by pipetting and their concentration was adjusted so that by seeding of test tubes with 0.6 ml approximately 60,000 cells were transferred. In this procedure the cell suspension was agitated by a magnetic stirrer and the volumes were distributed by means of an automatic pipette. Virus was added in 0.2 ml volumes to such tubes at one of three stages of incubation: (a) at the time of seeding ("O" cells); (b) after 1 to 2 days, when small islands of cells had formed; and (c) after 3 to 4 days, when sheets of cells ("S" cells) had developed. In the last case the concentration of horse serum in the medium was reduced to 10% at the time of infection. Cultures of monkey kidney epithelium were received from the Microbiological Associates, Inc. Either 150,000 or 300,000 cells had been seeded per tube in a medium consisting of 2% calf serum, 0.5% lactalbumen enzymatic hydrolyzate and 97.5% Hanks' solution. These cultures were inoculated after 5 days of incubation.

Experimental. In the first experiment sheets of HeLa cells ("S" cells) were inoculated with 0.2 ml of amniotic fluid of the 4th passage of the D. D. strain, which had been stored at –70°C for 10 months. After 24 hours of incubation lesions were seen particularly at the margins of the sheets. These consisted of large homogeneous masses of cytoplasm enclosing numerous nuclei and thus gave the appearance of multinucleated ("giant") cells. The same type of lesion was noted to varying extent with preparations of the other strains which had been stored at –70°C for periods up to 3 years. With fresh subcultures of these agents more extensive involvement of the sheets was seen, leading to such bizarre formations as seen in Fig. 1B.

Closer examination suggested that the formations did not represent multinucleated cells, but that the virus exerted a cytolytic effect which led to confluence of the cytoplasm of numerous cells. The nuclei, on the other hand, remained initially intact but were pushed together or lined up along the periphery of the cytoplasmic masses. This interpretation was confirmed by studying the development of the lesions. HeLa cells were seeded into Earle flasks. After 2 days, when small islands of cells had formed, the cultures were infected with the Barnes strain by addition of 0.2 ml of 2-fold diluted allantoic fluid to 2 ml of medium. Certain of the islands of cells were marked and photographed under the phase-contrast microscope at 1 to 2-hour intervals over a period of 24 hours. Representative stages in the development of the lesions in one island of cells over a period from 0 to 13 hours of incubation are shown in Fig. 2.

All pictures were taken under the same conditions of enlargement. It is immediately apparent that the island of cells increased considerably in diameter during the experimental

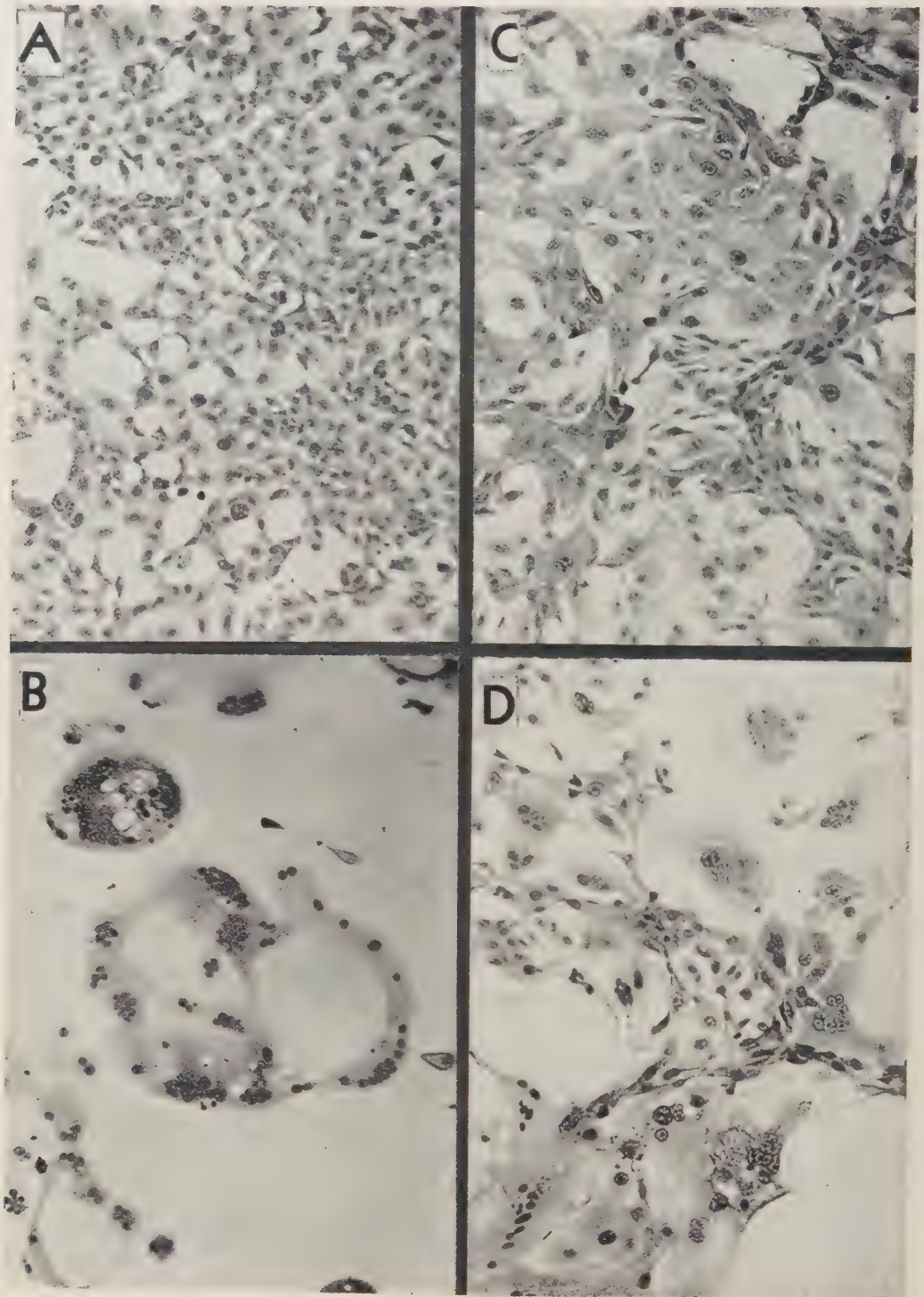

FIG. 1. Lesions produced by mumps virus (Barnes strain) in HeLa and monkey kidney cells (Giemsa stain, magnification 80×). (A) Normal HeLa cells; (B) HeLa cells 24 hr after addition of virus; (C) normal monkey kidney cells; (D) monkey kidney cells 48 hr after addition of virus.

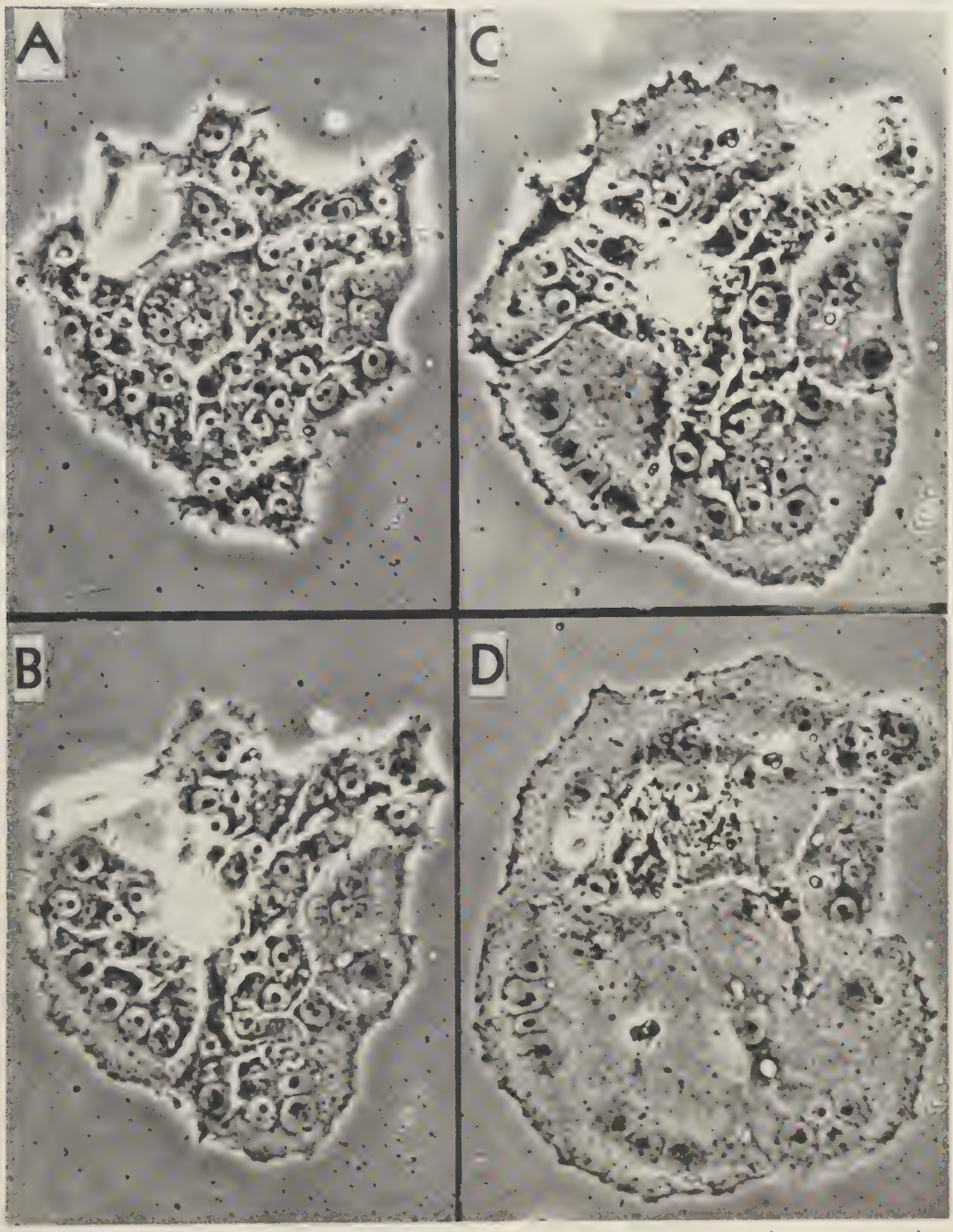

FIG. 2. Development of lesions in an island of HeLa cells (phase contrast microscope, magnification 80×). (A) The island before infection; (B-D) the same island 3, 6 and 13 hr after infection with Barnes strain of mumps virus.

period. The first changes were detectable 3 hours after infection (Fig. 2B). The cytoplasm along the edges of the island appeared to have been extruded and the outlines between cells had disappeared in places. With longer incubation these changes became more pronounced (6 hours, Fig. 2C) and finally individual cells were no longer discernible (13

TABLE I. Titration of Cytolytic Effect in HeLa Cells.

Virus					Cytolytic effect					
	Storage		ID_{50}/ml,	HA/ml,	Incuba-	Dilution of seed				
Strain	Temp., °C	Time, mo	log	log	tion, hr	1:2	1:4	1:8	1:16	1:32
Enders	—	—	8.5	3.11	24	4*	3	1	±	—
					48	4	3	1–2	1	—
	–70	2			12	3–4	2–3	1	0	0
					24	4	3	1	±	0
					36	4	4	2	±	0
	–20	3			24	4	2–3	±	0	0
					48	4	3–4	3	1	0
Barnes	—	—	7.8	3.41	24	4	4	2	1	—
	–20†	2			12	4	3–4	2	±	0
					24	4	4	3	1	±
					36	4	4	3	1	±
	–70	2			12	4	3–4	2–3	1	0
					24	4	4	3	2	±
					36	4	4	3	2	±
	–20	4			24	4	4	3	1	—

* Lesions were graded according to estimated percentage of cells affected. Thus 4 = about 100%; 3 = 75%; 2 = 50%, etc.

† This titration accompanied neutralization test shown in Table II.

hours, Fig. 2D). The nuclei appeared to be somewhat enlarged by the 3rd hour and definitely so by the 6th. After 13 hours several could no longer be seen and others showed evidence of caryolysis. In counting the nuclei it was apparent that they did not increase during the incubation period but rather decreased in number.

Inoculation of chick embryo - adapted mumps virus onto sheets of monkey kidney epithelium gave essentially similar results. Structures resembling multinucleated cells were readily discerned (Fig. 1D). Their size was on the whole smaller than seen with HeLa cells infected simultaneously with the same amount of virus. With the latter up to 50 nuclei could be counted in individual masses of cytoplasm, but with the former their number rarely exceeded 10. Furthermore, under optimal conditions few HeLa cells remained intact, whereas with the same inocula many strands of kidney cells survived, hemming in the lesions. These apparent differences may well be based upon the fact that the numbers of cells seeded into the tubes and the media employed differed in the 2 culture systems. In further experiments it was found that the cytolytic effect of mumps virus could readily be detected regardless of the stage of growth at which the HeLa cells were infected. It became apparent that the more cells were available ("S cells"), the smaller was the percentage affected, but the individual number destroyed seemed to be of a similar order as with "O" cells, infected at the time of seeding. With "S" cells additional effects were seen, consisting of groups of shrunken, degenerated cells, with small, pycnotic nuclei. For convenience "O" cells were employed for further analysis of the quantitative aspects of the cytolytic reaction. As can be seen in Table I, cytolysis was noted only with large concentrations of virus. The majority of cells were affected within 48 hours when the infected allantoic fluids were diluted 1:4 or 1:8, but little or no effect was noted when the inocula were diluted more than 16-fold. The cytolytic titers of the seeds remained the same after storage of the virus at –20°C for at least 4 months. Titrations in sheets of monkey kidney cells gave similar endpoints, although the extent of the lesions was less pronounced. Since the seeds contained over 10^7 infectious doses for chick embryos per ml and the cultures harbored only about 10^5 cells, it is readily evident that numerous virus particles were required per cell in order to produce cytolysis.

It is also seen in Table I that the partial

lesions obtained with the more dilute inocula of the allantoically-adapted strains of virus often did not progress significantly upon prolonged incubation. This suggested that the process was incapable of spreading from the cells originally affected to the remaining normal ones. In line with this observation is the fact that the cytolytic effect could not as yet be maintained on serial passages in HeLa cells of culture medium alone, or of the combined medium and cells. Cytolytic effects were noted upon second passage of materials derived from completely destroyed cultures (initial inoculum of undiluted allantoic fluid seed), but on 3rd transfer such results were no longer observed. If the passage series were initiated with smaller amounts of seed virus the second transfer revealed no lesions. These data do not necessarily denote absence of viral propagation and further passage experiments are in progress. In more recent experiments (5) with strains of mumps virus which had been recently isolated and had undergone only a few amniotic passages (Tucker and D. D. strains), some cytolytic effect was seen when the amniotic fluids were diluted as high as 1:10,000. Furthermore, evidence of adaptation to HeLa and monkey kidney cells has been obtained in that cytolytic lesions were seen still after 6 to 12 transfers in tissue culture. These results will be reported at a later date.

It has been pointed out that cytolytic effects were obtained with strains of virus which had been stored in the frozen state for several years. It is likely that under these conditions the infectivity of the preparations had decreased to some extent. This suggested that the lytic property possibly was more stable than infectivity. Indeed, when dialyzed, infected allantoic fluids were exposed to ultraviolet irradiation to the extent that the infectivity for chick embryos was destroyed the cytolytic activity in HeLa cells was reduced only by a factor of 4. Thus, it became evident that the cytolytic and infectious properties of the virus were not identical.

The cytolytic effect of mumps virus was readily neutralized by human mumps convalescent or post-vaccination sera, but to a lesser extent or not at all by sera taken during the acute stage of the illness or prior to immunization. Paired sera derived from patients with other infectious diseases failed to show rises in anti-cytolytic activity. For convenience, the neutralization tests were set up in the following manner: Serial 2-fold serum dilutions were made in culture tubes in 0.2 ml volumes. To these were added 0.2 ml of the virus preparation adjusted to contain between 2 and 4 cytolytic units, based upon a 2^+ endpoint. After incubation for 1 hour at 37°C, 60,000 HeLa cells were added by automatic pipette as described. The tubes were shaken and then incubated at 37°C. The tests were read after 12, 24 and 36 hours of incubation. Representative results are shown in Table II.

Discussion. It is evident from the data presented that mumps virus produces lesions in HeLa and monkey kidney epithelial cells, which superficially resemble "giant cells." This type of lesion is different from those noted with other agents, with the possible exception of measles virus(6). In the case of mumps virus it has been shown that the lesions do not represent multinucleated cells but are rather the result of cytolysis and confluence of the cytoplasm. Nuclear changes become evident only late in the incubation period. This cytolytic effect is produced by established laboratory strains as well as by recently isolated mumps virus. The available evidence indicates that cytolysis is seen only when the concentration of virus particles far exceeds the number of cells present in the culture.

It is of interest to note that mumps virus belongs to the group of agents which may cause hemolysis(7). The hemolytic titers of mumps virus preparations reported in the literature are similar in order to those obtained in assays of cytolytic activity. On exposure to ultraviolet light the hemolytic activity is far less readily affected than infectivity(8) and the present data reveal a similar discrepancy between the inactivation of the cytolytic and infectious properties. Upon dialysis of infected allantoic fluids hemolysis increases(9) and preliminary experiments have indicated that cytolysis likewise is enhanced. Furthermore, Newcastle disease virus, which also pos-

TABLE II. Neutralization of Cytolytic Effect of Mumps Virus (Barnes Strain) by Sera from 2 Patients, Taken during Acute and Convalescent Phases of Parotitis.

Days after onset	Serum Complement fixation titer* Anti-S	Anti-V	Dilution of virus	Incubation (hr)	Cytolytic effect (HeLa cells) Dilution of serum 1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	Titer†
					Patient No. 978								
2	<1:2	<1:2	1:2	12	4	4	4						<1:2
				24	4	4	4						"
				36	4	4	4						"
			1:4	12	3	4	4						"
				24	4	4	4						"
				36	4	4	4						"
11	1:32	1:64+	1:2	12	0	0	0	0	0	±	1	3	1:192
				24	0	0	0	0	±	1	3	3	1:96
				36	0	0	0	+	2	3	4	4	1:32
			1:4	12	0	0	0	0	0	0	±	2	1:256
				24	0	0	0	0	0	±	1	3	1:192
				36	0	0	0	0	0	±	1	3	"
					Patient No. 888								
2	1:4	1:8	1:2	12	0	1	2	4					1:8
				24	1	2	4	4					1:4
				36	1	2	4	4					"
			1:4	12	0	±	1	2					1:16
				24	±	1	3	4					1:6
				36	±	1	3	4					"
17	1:16	1:64	1:2	12	0	0	0	0	±	2	3	3	1:64
				24	0	0	0	1	2	3	4	4	1:32
				36	0	0	0	1	3	4	4	4	1:24
			1:4	12	0	0	0	0	0	±	1	3	1:192
				24	0	0	0	0	±	1	2	3	1:128
				36	0	0	0	0	1	2	3	3	1:64

* S = soluble; V = virus antigens. † Based upon 2+ lesion endpoint.

sesses hemolytic activity(10), was found to yield cytolytic lesions in addition to other cytopathogenic effects in HeLa cells(11). On the other hand, influenza viruses, which are non-hemolytic, failed to induce cytolysis. These considerations suggest that the cytolytic effect of mumps virus may well be related to the hemolytic property.

Thus far it has not been possible to transmit the cytolytic effect serially either in HeLa or monkey kidney cells when the series has been initiated with allantoically adapted strains of virus. This does not necessarily imply that the virus does not propagate in these cultures. It may indicate merely that the concentrations of virus reached are too small to induce cytolysis since, as has been pointed out, large quantities are needed to cause this effect. On the other hand, recent observations indicate that strains of mumps virus, freshly isolated by the amniotic route, continue to produce lesions on serial passages in both types of cultures (5). These facts suggest that upon extensive adaptation to the chick embryo the capacity of the virus to propagate in human or simian cells becomes impaired. Indeed, attenuation of mumps virus upon prolonged passage series in the chick embryo has been demonstrated by its failure to induce overt disease in susceptible children(12,13), while early amniotic passages caused disease(14). These problems are being investigated further.

Summary. Upon inoculation of cultures of HeLa or monkey kidney epithelial cells with large amounts of several strains of mumps virus at various stages of chick embryo-adaptation lesions resembling "giant cells" were observed, which however were shown to be caused by a cytolytic property of the agents. This effect could not be maintained on serial passage when allantoically-adapted strains were employed. Cytolysis was specifically prevented by human mumps convalescent and post-vaccination sera but not, or to a lesser

extent, by sera taken during the acute stage of the disease or prior to immunization. The cytolytic activity was found to be more resistant to ultraviolet light than the infectious property. The possible relation of the cytolytic effect to the hemolytic activity of the virus is discussed.

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A New Anticholinesterase Oxamide. (21391)

Aaron Arnold, Albert E. Soria, and Fred K. Kirchner.
(Introduced by M. L. Tainter.)
From the Sterling-Winthrop Research Institute, Rensselaer, N. Y.

In an earlier publication from this Institute(1) the curarimimetic properties of a series of amino- and ammonium-alkylaminobenzoquinones was discussed. The most promising of these [Mytolon, Win 2747(2)] was observed subsequently to have its use limited by its parasympathomimetic properties evidenced clinically by sialorrhea(3). Since the parasympathomimetic properties appeared to be associated with high anticholinesterase activity, this property of the amino- and ammonium-alkylaminobenzoquinones was studied(4). During the course of these studies it was observed that substitution on the benzene ring of the benzyl quaternizing group imparted unusual anticholinesterase properties to the benzoquinones. While this observation has not yet enabled us to develop benzoquinones of clinical interest, it has been applied to another series of acetylcholinesterase inhibitors with the synthesis apparently of clinically useful compounds.

It has been observed that some quaternized amino alkyl amides of the dicarboxylic acids have an anticholinesterase activity only moderately lower than that of neostigmine methyl sulfate. Of direct interest is the observation that one of them, N,N′-bis(2-diethylaminoethyl)oxamide bis-2-chlorobenzyl chloride (Win 8077), on a molar basis, is about 6 times as effective as neostigmine methyl sulfate as an anticholinesterase. The potential interest in this compound is borne out by the fact that pharmacological(6) and clinical investigations (7) on it for the amelioration of the symptoms of myasthenia gravis offer a basis for the belief that it will prove clinically useful.

Methods. The inhibition of hemolyzed red cell cholinesterase by various compounds was studied by the electrometric titration procedure of Michel(5). By adding the hemolyzed cells in 0.5 cc volume, rather than the 1.0 cc volume suggested by Michel, it was possible to add the inhibitor (or water) in 0.5 cc volume without modifying the 2.2 cc total volume employed by Michel. The point of 50% inhibition was estimated by plotting on logarithmic probability paper the amount of inhibitor used against the amount of inhibition noted.

Results. The results of the tests for the

TABLE I. Relative Anticholinesterase Activity of Neostigmine Methyl Sulfate and Some Substituted Oxamides.

Win No.	Formula	Amt for 50% inhibition: μg/2.2 ml reaction mixture	Amt for 50% inhibition: Molar conc.
—	$3\text{-}(CH_3)_2NCOOC_6H_4N(CH_3)_3 \cdot CH_3SO_4$*	.18	2.45×10^{-7}
3286	$(\text{-}CONH(CH_2)_2N(C_2H_5)_2 \cdot C_6H_5CH_2Cl)_2$	8.15	6.86×10^{-6}
8032	$(\text{-}CONH(CH_2)_2N(C_2H_5)_2 \cdot 4\text{-}ClC_6H_4CH_2Cl)_2$	2.35	$1.75 \times$ "
8077	$(\text{-}CONH(CH_2)_2N(C_2H_5)_2 \cdot 2\text{-}ClC_6H_4CH_2Cl)_2$	.0565	4.2×10^{-8}
8078	$(\text{-}CONH(CH_2)_2N(C_2H_5)_2 \cdot 2\text{-}CH_3OC_6H_4CH_2Cl)_2$	2.35	1.78×10^{-6}
8626	$(\text{-}CONH(CH_2)_3N(C_2H_5)_2 \cdot 2\text{-}ClC_6H_4CH_2Cl)_2$	1.75	$1.25 \times$ "
8702	$(\text{-}CONH(CH_2)_2N(CH_3)_2 \cdot 2\text{-}ClC_6H_4CH_2Cl)_2$	10	$7.9 \times$ "
12306	$(\text{-}CONH(CH_2)_2N(C_3H_7)_2 \cdot 2\text{-}ClC_6H_4CH_2Cl)_2$	2.15	$1.47 \times$ "

* Neostigmine methyl sulfate.

anticholinesterase activity of various Win compounds in comparison with that of neostigmine methyl sulfate are summarized in Table I. From the table it may be seen that N,N′-bis(2-diethylaminoethyl)oxamide bis-2-chlorobenzyl chloride (Win 8077) is about 6 times as effective as neostigmine methyl sulfate as an anticholinesterase. It is seen, also, that minor changes in the structure of Win 8077 reduced the anticholinesterase activity. When the quaternizing agent was 4-chlorobenzyl chloride (Win 8032) or 2-methoxybenzyl chloride (Win 8078) in place of the 2-chlorobenzyl chloride of Win 8077, the anticholinesterase activity was 1/7 of neostigmine methyl sulfate. With the benzene ring of the benzyl chloride quaternizing agent was otherwise unsubstituted (Win 3286), the anticholinesterase activity was 1/30 that of neostigmine methyl sulfate. A decrease in the alkyl substitutions on the terminal nitrogens of Win 8077 from diethyl to dimethyl (Win 8702) decreased the anticholinesterase activity to 1/30 that of the reference material. An increase in the alkyl substitutions on the terminal nitrogens of Win 8077 from diethyl to dipropyl (Win 12,306) decreased the anticholinesterase activity to 1/6 that of neostigmine methyl sulfate. Lastly, an increase in the distance between the amide and terminal nitrogens from $-CH_2CH_2-$ in Win 8077 to $-CH_2CH_2CH_2-$ (Win 8626) yielded an oxamide with 1/5 the activity of neostigmine methyl sulfate. The quaternized amides of related dicarboxylic acids likewise were less active cholinesterase inhibitors than the reference material. While the interest of de Beer *et al.*(8) was primarily in the effect on myoneural blocking, these authors noted that their succinic, glutaric, and adipic amides possessed greater physostigmine-like activity than their oxalic and malonic amides. The difference in results of de Beer *et al.* and those given above probably resulted from the differences in the quaternizing groups.

Summary. Studies on amino- and ammonium-alkylaminobenzoquinones directed our attention to the unique property of the quaternizing agent, 2-chlorobenzyl chloride, in increasing their anticholinesterase activities. This was applied to amides of dicarboxylic acids, and N,N′-bis(2-diethylaminoethyl)oxamide bis-2-chlorobenzyl chloride (Win 8077) was synthesized. It was observed to be about 6 times as effective as neostigmine methyl sulfate as an inhibitor of red cell cholinesterase.

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Effect of Partial Starvation on Development of Diabetic Cataracts.* (21392)

JOHN W. PATTERSON.

From the Department of Anatomy, School of Medicine, Western Reserve University, Cleveland, O.

Diabetic cataracts are related to severity of diabetes(1,2). Hyperglycemia, determined by multiple analyses in the non-fasted animal, serves as an indicator of the severity of the condition. The rate at which cataracts develop varies directly with the height of the blood sugar(1). Lowering the blood sugar by administering phlorizin(3) or by feeding a high fat diet(2,4) prevents the occurrence of cataracts. It has been suggested that the high levels of glucose may competitively block the absorption by the eye of some essential metabolite, and thus produce a nutritional deficiency(5). This hypothesis would be substantiated if dietary supplements could be used to prevent diabetic cataracts. If the hypothesis is correct, such supplements should be administered at a time when the blood glucose level is low so that there would be a minimum of competition for absorption by the eye. Supplements administered without glucose during a period of starvation might achieve this condition. It is the purpose of this paper to present the effects of partial starvation on the development of cataracts.

Experimental. A total of 38 Sprague-Dawley rats weighing 100-120 g were injected with 40 mg per kilo of alloxan monohydrate. Twelve of the rats either failed to become diabetic or died of diabetes. The remaining 26 diabetic rats were divided into two groups. One group consisting of 7 rats served as a control to check the curve depicting the relationship between hyperglycemia and the development of diabetic cataracts which was previously determined in this laboratory(3). These rats were allowed to eat commercial dog chow (Friskies) and drink water as they desired. The second group consisting of 19 rats were treated in the same manner as the control group for the first 3 weeks. Thereafter they were starved one day each week. The food was taken away on the previous evening and returned the following morning making a total of approximately 40 hours of starvation each week. Blood sugars were determined twice each week in both groups(6). These determinations were timed so that in the starved group one determination would fall on the day of starvation and the second determination on a day on which the rats were fed. The animals were followed for a minimum of 21 weeks.

During this period 10 rats died without developing cataracts. The 9 remaining rats provide the data for this paper. These rats were observed each day for cataracts as previously described(1).

The 7 control rats had blood sugars of 440-510 mg per 100 cc of blood with an average of 480 mg per 100 cc. They developed cataracts in an average time of 70 days with a standard deviation of 8 days. The control group thus fell on the previously determined diabetic cataract curve which is depicted in Fig. 1.

The 9 rats that had been starved for 40 hours each week were divided into 2 groups based on the non-fasting blood sugar. There were 5 rats with average blood sugars between

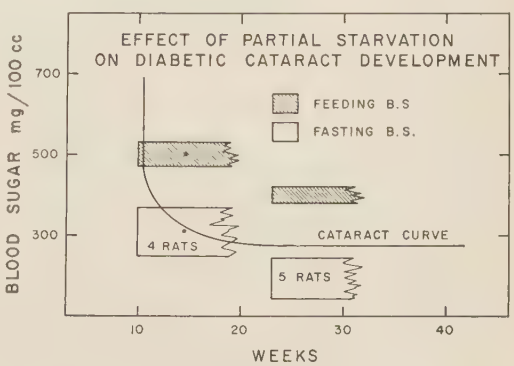

FIG. 1.

* This investigation was supported by a research grant from the National Institute of Arthritis and Metabolic Diseases, of the National Institutes of Health, Public Health Service. The author wishes to express his appreciation for this support and also for the technical assistance of Miss Annette Charlillo.

350 and 450 mg per 100 cc and 4 rats with blood sugars between 450 and 550 mg per 100 cc. During the starvation period the blood sugar of the group with the more severe diabetes dropped from an average of 500 mg per 100 cc to an average of 310 mg per 100 cc. Two rats developed cataracts in 81 days, one rat in 96 days and one failed to develop cataracts in 21 weeks. The average time for cataract formation is thus 14.5 weeks which is approximately what would be expected on the basis of the blood sugar level during the starved period. In the group with the less severe diabetics, the blood sugar was lowered from an average of 400 mg per 100 cc to an average of 195 mg per 100 cc during the starvation period. None of these rats developed cataracts in the 21 week period. Four of the 5 rats were followed for a longer time and and at the end of 30 weeks, none had developed cataracts. Non-starved diabetic rats with this severity of diabetes developed cataracts in 11.3 weeks with a standard deviation of less than 3 weeks. The complete prevention, therefore, of cataracts for 21 to 30 weeks is highly significant. (Fig. 1).

Discussion. Lowering the blood sugar of diabetic animals by partial starvation prevents cataracts to the extent that the blood sugar is lowered. These results are in agreement with those obtained by lowering the blood sugar with phlorizin(3) or a high fat diet(2, 4), and can be interpreted as indicating the direct role of hyperglycemia in the production of cataracts. It is interesting to note, however, that these 3 methods of preventing cataracts produce gross changes in the body metabolism with increased levels of fat or ketone bodies in the blood. Since the eye is dependent on the nutrients in the blood for its energy, this change in the ratio of energy yielding compounds suggests an alternate mechanism for explaining the production of cataracts. If diabetic cataracts are due to a lack of insulin with a loss in the ability to utilize glucose for energy, then the provision of an alternate energy supply from ketone bodies or fat might raise the total available energy above the critical level which is necessary for the prevention of cataracts. This alternate explanation is consistent with recent work(7) which demonstrates that, unlike galactose, high blood levels of glucose do not affect the eye directly.

Starvation for a period of 40 hours a week prevents cataracts. The effectiveness of this short period perhaps explains why diabetic cataracts are not observed more frequently in juvenile human diabetes. If starvation prevents cataracts by producing ketosis it should be quite effective in man who is more prone to produce ketones than is the rat. The effectiveness of starvation in preventing cataracts makes it difficult to test the value of different supplements at a time when the blood sugar is lowered. However, if the lowering of the blood sugar prevents cataracts by providing an alternate supply of energy, it should be possible to prevent diabetic cataracts with the proper supplements while the level of blood glucose is high. It may be possible to determine what these substances are by studying the effect of supplements in the prevention of galactose cataracts. The latter cataracts are similar to diabetic cataracts in appearance (8) and in the fact that each condition potentiates(5) the other in the production of cataracts.

Summary. Lowering the blood sugar of diabetic rats by starvation for a period of 40 hours each week prevents cataracts to the extent that the blood sugar is lowered. Starvation produces a drop of approximately 200 mg of glucose per 100 cc of blood. Cataracts are prevented if the severity of diabetes is such that the non-fasting average blood sugar is 450 mg per 100 cc or less.

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Salmon Poisoning Disease of Canines. II. Further Observations on Etiologic Agent. (21393)

Cornelius B. Philip, Lyndahl E. Hughes, Betty Locker, and W. J. Hadlow.
(Introduced by Carl L. Larson.)

From the U. S. Department of Health, Education, and Welfare, Public Health Service, National Institutes of Health, National Microbiological Institute, Rocky Mountain Laboratory, Hamilton, Mont.

In the first report, the authors(1) proposed the name *Neorickettsia helminthoeca** for a rickettsialike agent(2) of "salmon poisoning disease" of dogs transmitted by a trematode, *Nanophyetus salmincola* (Chapin). The present report offers additional observations on transmission and etiology including attempts to obtain information on the extra-vertebrate phases in snails and in fluke eggs. Passages of fresh or frozen mesenteric node tissues of infected dogs for tests or challenges were made by intraperitoneal injection of standardized inocula containing 5 ml of 20% suspensions in physiological salt solution. Infection in all instances was confirmed by gross and microscopic pathology or by challenge of recovered animals.

Experimental. We infected 61 dogs either in primary tests or by challenge. No instance of suspected natural resistance has been encountered. All but 3 dogs were kennel-raised beagles. Of 32, in which neither sacrifice before death nor antibiotic treatment intervened, only 4 spontaneously recovered. Of these 4, only one received a standard exposure. The feeding of trout fingerlings of various sizes containing fluke cysts from 7 separate iced shipments from an Oregon fish hatchery (as previously(1), by courtesy of G. C. Webb) has resulted in 11 infections in dogs and only two failures in 20 months, thus showing high degree of infectivity of this source.

Susceptible dogs fed fish, or, injected with suspensions from frozen nodes, had longer average incubation periods (to but excluding first day of fever, usually of 39.6°C or higher) than animals injected with freshly harvested nodes. Of 50 dogs under routine test, 90% of the 27 injected with frozen node or fresh adult fluke suspensions as well as feeding of fluke-infested fish, had incubation periods of 6-7 days. In contrast, 95% of 23 others which received fresh node suspensions had incubation periods of 2-4 days. Additional evidence that there may be a quantitative relationship between dosage and incubation period is afforded in the only titration experiment thus far attempted. Donor dog no. 52 was infected by feeding of fluke-infested fingerlings. A 20% suspension in sterile milk of its nodes taken at sacrifice on the first post-febrile day, was titrated in 0.2% bovine albumin in buffered saline. Each of 5 weanling (60-day-old) beagles of the same litter received 5 ml of respective ten-fold dilutions of $10^{-2.7}$ to $10^{-6.7}$. All were fatally infected but showed increasing incubation periods apparently correlated with increasing dilution as shown in Table I. Considering the long, 19-day incubation period of the last dog, it is problematic if dilution by another log would have been infectious.

However, there is apparent variation in individual reactivity if incubation period is a criterion, as illustrated by use of fresh node suspensions of dog no. 31 in replicate passage

TABLE I. Periods of Incubation, Fever, and Duration to Death in Five Young Beagle Dogs Injected with Graded Doses of Infectious Node Suspensions of *Neorickettsia helminthoeca.*

Period in syndrome	Days duration for respective 10-fold dilutions in each of 5 littermate dogs				
	$10^{-2.7}$	$10^{-3.7}$	$10^{-4.7}$	$10^{-5.7}$	$10^{-6.7}$
Incubation	3	5	7	7	19
Fever	4	4	4	4	3
Defervescence	10	5	5	5	5
Total until death	17	14	16	16	27

* Originally helminthēca; typographical dropping of the long "e" causes confusion of the etymology of this epithet and we are returning to the preferred though less euphonius transliteration of Greek "oi" to Latin "oe" (see *Int. Bull. Bact. Nomen. Tax.*, 3:67, 1953).

to 8 other dogs. One each developed fever on second and third days, 5 on fourth, and one on seventh day.

Evidence that the fluke is an intermediate host is strengthened by the following. Dog no. 13 recovered spontaneously after 8 days of severe febrile illness following feeding of a fresh fingerling. It was refed other Oregon fish twice, up to 45 days after initial feeding, without further febrile reaction. It was then sacrificed 46 days after last feeding (or 81 days after defervescence). Approximately 350 adult flukes were recovered from the small intestine, washed and injected into dog no. 24 which developed a severe infection and sacrificed for further passage. Dog no. 25 received at the same time fresh node suspension from no. 13 without becoming infected and susceptibility of dog no. 25 was confirmed 42 days later by challenge with stored nodes of no. 24. This observation appears to confirm that infection may be residual in trematodes while not demonstrable in node tissues of recovered host. Furthermore, dog no. 61 developed a characteristic fatal infection following injection with some 500 flukes recovered at sacrifice on nineteenth day from a fish-fed, symptomless, Florida raccoon, which has been relatively refractory to this disease in our experience, as will be reported elsewhere. Infection in adult flukes recovered at autopsy of fluke-infected dogs has also been confirmed by positive results in 2 other instances after several washings of the flukes in saline and incubation at 37°C in physiological salt solution for 24 hours; in a third test in which harvested flukes were refrigerated at minus 16°C for 30 days, one day of fever resulted on eighth day after injection and the animal resisted later challenge with controlled node material. A loss in potency had obviously occurred in this instance.

In contrast to demonstrated survival of infection in 24-hour stored living flukes from infected dogs, is the result of an attempted filtration test with a suspension in physiological salt solution of triturated flukes from an infected dog which was invalidated because the agent was not even persistent in the unfiltered control through the 2 hours required for the operation at room temperature. It is increasingly apparent that *N. helminthoeca* is not a very resistant agent under various conditions of stress. Tests for infection in fluke eggs have not been reported. Eggs were recovered from flukes harvested at necropsy that have been incubated at 37°C in saline for over 48 hours. Dog no. 22 received intraperitoneally a suspension in heart broth infusion of a calculated 14,000 eggs which had been carefully separated from flukes, then stored at room temperature for one month with changes of tap water, and which appeared viable when used. Dog no. 41 was similarly injected with an estimated 51,000 eggs harvested and washed, representing an accumulation from harvested flukes incubated at 37°C during 48 hours. Brief chilling at room temperature in this instance occurred only during recovery of the adult flukes and the later preparation of the ground-egg suspension in 0.2% beef albumin in buffered saline. Neither dog reacted and both died of the disease after challenge on the twenty-seventh and twenty-eighth days. The dogs from which the flukes were harvested had had characteristic infections, confirmed both clinically and pathologically. No records of recovery of infection from fluke cercariae in snails have been published. Although dissection of mature *Goniobasis* snails obtained from the endemic area in Oregon has revealed heavy parasitism by immature flukes of several species, only a few snails in our shipments could be confirmed as having infestations with the distinctive, stub-tailed *Nanophyetus* cercariae(3). Three dogs have been injected respectively with suspensions of 4, 2, and 1 (plus 4 probables) of such parasitized Oregon snail livers without developing infection and were susceptible to challenge 48 to 85 days later. A fourth dog (no. 28) which received 2 other *Nanophyetus*-infested livers collected by one of us (B.L.) on the Clackamas River, Oregon, developed 3 days of fever 28 days after injection and was sacrificed for passage on the 34th day. It was never in contact with an infected dog. A fifth dog (no. 66) injected with 3 such livers of snails from the original Alsea River area†

† Thanks are due Mr. Darrell Darrow of the Rocky Mountain Laboratory, for collecting these snails for us.

developed a characteristic clinical and fatal infection after an even longer incubation period of 33 days. This animal was in a clean cage by itself the entire time. The pathologic picture in both was characteristic including positive node imprints, and the infection was maintained for 2 passages from no. 28 in other dogs which had shortened incubation periods of 7 and 4 days. Unless the long incubation periods in both original tests were due to reduced virulence or low doses, they might suggest that these were kennel infections by some unknown means. However, we have had a number of eventually-proved susceptible dogs pending test (including litters and dogs awaiting challenge) penned with sick dogs because of shortage of space. As in the long experience of Simms *et al.*(4), no other possible contact infections occurred for which we could not account. Nevertheless, these 2 observations will need to be repeated before it can be decided whether *N. helminthoeca* is infectious in the snail phases of the fluke cycles.

We have confirmed Cordy and Gorham's(5) demonstration of the susceptibility of the etiologic agent to certain antibiotics, as are other Rickettsiaceae, and terramycin has been added to the effective list. As few as 2 capsules orally of 250 mg each of aureomycin or terramycin, 8 hours apart during the febrile episodes, has produced defervescence in 15-lb beagles within 24 hours with dramatic subsidence of symptoms and resumption of feeding. In a further series, 3 untreated dogs died of 5 which were injected with replicate doses of fresh, infectious node suspension. Treatment of the other 2 was reduced to but one capsule of 250 mg each of aureomycin and terramycin, respectively, on the fourth day of severe febrile illness in each. Both resumed eating within 48 hours and recovered in contrast to the above 3 fatal controls. In another instance, administration of terramycin in a divided dose of 500 mg on the fourth day of fever was followed by prompt objective improvement in contrast to 4 untreated controls, all of which terminated fatally. Terramycin totalling 875 mg initiated on the third day of fever and given orally in divided doses during 3 days did not destroy the organisms in the nodes of infected dog no. 39 which was sacrificed on the sixth day of defervescence. Dog no. 46 developed a typical course following an incubation period of 7 days after injection with suspension of these nodes. Only one apparent relapse in 7 dogs treated with varying doses of antibiotics has been observed, and that consisted of a new febrile episode of 7 days with recurrence of anorexia one week (afebrile) after administration of only 250 mg of aureomycin. Further treatment was not instituted, however, and improvement was slower than in other treated animals.

Because of similarities of the organisms to *Colesiota* rickettsia-like agents causing conjunctivitis in South African animals called to attention by Coles(6), 0.5 ml of proved infectious dog node suspension was instilled in the conjunctival sac of one eye of dog no. 35 without causing infection and the dog proved susceptible 53 days later. Nevertheless, conjunctival lesions have been observed by us incidental to other routes of infection as will be reported elsewhere.

The authors have previously assigned this agent to the bacterial family Rickettsiaceae and have indicated its distinctness from *Ehrlichia canis* (Donatien and Lestoquard).

Summary. To elucidate the method of persistence of the rickettsialike agent of salmon poisoning disease of dogs, *Neorickettsia helminthoeca,* the authors failed to infect dogs in 2 tests by injection of eggs laid by flukes from infected dogs. In 5 tests by injection of dogs with fluke stages from dissected livers of Oregon snails, 3 resulted negatively, while in 2 others, the dogs developed characteristic severe infections, confirmed pathologically, after unusually prolonged incubation periods. Flukes and node tissues from antibiotic-treated, recovered dogs, and flukes from a refractory raccoon were shown to remain infectious. As little as 250 mg (one commercial capsule at one feeding) of aureomycin or terramycin were shown to save 15-pound beagles when given as late as the fourth day of fever compared to fatal infections in controls. In one titration experiment, node suspension was found to be infectious in a dilution of $10^{-6.7}$. Although the agent has been assigned to the

Rickettsiaceae, it appears generically distinct from *Ehrlichia canis* and *Colesiota* spp.

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Preparation of Highly Purified Intrinsic Factor.* (21394)

WILLIAM L. WILLIAMS, LEON ELLENBOGEN, AND RAYMOND G. ESPOSITO.
(Introduced by T. H. Jukes.)

From Nutrition and Physiology Section, American Cyanamid Co., Research Division, Lederle Laboratories, Pearl River, N. Y.

Several investigators have achieved partial purification of intrinsic factor by applying one or two fractionation steps. The present paper describes the further purification of intrinsic factor from an ammonium sulfate precipitated fraction of which 13 mg was an effective daily dose. A more homogeneous fraction was obtained of which 1 to 2 mg was an effective dose. All tests for activity were made using pernicious anemia patients in relapse. The tests were considered valid only if the patient failed to respond to 10 to 20 γ of vitamin B_{12} per day for 10 days before giving vitamin B_{12} plus intrinsic factor and if the reticulocyte peak was near the average expected according to Sturgis and Isaacs(1) for parenteral therapy of pernicious anemia with liver extract.

Experimental. A method to obtain an ammonium sulfate (AS) fraction apparently similar to fraction B of Prusoff *et al.*(2) was developed which could be applied to dried hog stomach. Hog stomach powder which was desiccated and defatted at low temperature was selected as starting material since apparently the effective daily dose of selected lots is 1 to 4 g as compared to 50 g for the processed hog stomach powder used by Prusoff *et al.* The physical nature of our hog stomach preparation necessitated the use of strong centrifuging rather than filtration to collect all extracts and precipitates. We used the same amounts of ammonium sulfate as Prusoff *et al.* corresponding to approximately 35 and 55% saturation but the temperature varied from 10° to 13°C instead of 4°. Twenty to 30 g of the AS fraction was obtained from 8 kg of dried and desiccated hog stomach. An average yield of 3 g of AS fraction per kg of dried hog stomach was obtained compared to 0.3 g per kg obtained by Prusoff *et al.* Both products had similar potency of about 15 mg per effective daily dose. The *AS fraction* was further fractionated by the following procedure: Twenty g of the dialyzed AS fraction was dissolved in 210 ml of distilled water. After the solution was adjusted to pH 7.6 by 4 N KOH, 200 mg of crystalline trypsin and 200 mg of crystalline chymotrypsin were added and the mixture incubated for 2 hours at 37.5°C with occasional shaking. After digestion the solution was adjusted to pH 5.0 with 4 N HCl and then dialyzed in cellophane bags overnight against cold running tap water. The pH was adjusted to 6.3, the solution chilled to 3°C and cold 95% ethanol was slowly added with stirring over a

* We are greatly indebted to Dr. T. D. Spies, Nutrition Clinic, Hillman Hospital, Birmingham, Ala., and to Dr. R. Janet Watson and Dr. Herbert Lichtman, Kings County Hospital, Brooklyn, N. Y., for clinical evaluation of the various fractions. Dr. R. A. Brown of these Laboratories gave valuable advice and carried out the physical chemical measurements and interpretations.

period of 1 hour until the final alcohol concentration of the solution was 40% by volume.

During the addition of the alcohol the temperature was gradually lowered to –6°C and maintained at this temperature for an additional 1.5 hours after all the alcohol had been added. The solution was then centrifuged in a Sharples centrifuge at 4°C. The resulting precipitate was discarded and the supernatant chilled to 0°C. Ethanol was added over a period of 1.5 hours to bring the alcohol content of the supernatant to 80% by volume. During the addition of the alcohol the temperature of the solution was gradually lowered to –6°C and maintained at this temperature for an additional 1.25 hours. The solution was then centrifuged as above and the resulting supernatant liquid discarded. The precipitate was then suspended in 30 ml of distilled water and dialyzed overnight. The contents of the cellophane bag were then subjected to ultrafiltration for 40 hours through a parlodion membrane prepared with acetic acid and ether according to Bauer(3). The ultrafiltration residue (UFR fraction) was freeze-dried. The 40% ethanol precipitate and the fraction passing through the parlodion membrane were discarded on the basis of negative clinical tests. Twenty and 40 g quantities of AS fraction (11 - 12% nitrogen) were fractionated according to the above procedure, the distribution of nitrogen and hence solids for a 20 g batch being in most instances as follows: After digestion half the solids passed through the dialysis bag leaving a 10 g residue. Alcohol fractionation yielded 5 g of active fraction precipitating at 80% alcohol. During the alcohol treatment low molecular weight material developed which was found to pass through a parlodion membrane and to be inactive. Various UFR fractions weighed between 1 and 2 g.

Results. Forty-eight kg of dried and defatted hog stomach were processed in 8 kg quantities by the above described procedure to obtain 120 g of AS fraction. Through the cooperation of Dr. Arnold Welch, this batch of AS fraction was tested and found to possess definite clinical activity at 15 and 60 mg per day (patients G.K. and W. C. in Prusoff *et al.* (2)). A second lot of AS fraction was prepared from the same starting material and further fractionated to yield UFR fractions. Because only a small quantity of material was prepared initially, the first UFR fraction was administered for a short time to four pernicious anemia patients in relapse. With all 4 patients, definite activity was exhibited at a dosage of 1.1 mg per day. From this second lot of AS fraction larger quantities of UFR fractions were prepared and given to two patients at 1 mg per day for 21 days with the results shown in Table I. The dosage of 1 mg/day of UFR fraction was apparently below optimum since the observed reticulocyte peaks in these 2 patients were submaximal. However, from the partial reticulocyte responses and marked clinical improvement of the 2 patients, there is no doubt of the intrinsic factor content of this UFR fraction.

TABLE I. Clinical Tests on a UFR Fraction.*

Patient	Red blood cell count		Reticulocyte peak	
	Initial	At 21 days	Observed, %	Expected(1), %
	million/mm³			
S.A.	2.36	2.71	9.2 (10th day)	17
M.E.	2.41	2.86	8.6 (10th day)	16.5

* Tests carried out by Dr. T. D. Spies. Patients were given 10 γ of vit. B_{12} daily for 10 days and then received 10 γ daily with 1 mg of UFR fraction.

A third large lot of AS fraction was prepared to serve as a constant source of UFR fraction. The results of clinical tests of this AS fraction and one of the UFR fractions prepared from it are described in Table II. The UFR fraction, No. 21-2, was fully active at 2 mg per day and possibly at a lower dosage. Patient E. K. showed submaximal reticulocytosis, however, after therapy was terminated at 21 days, the red blood cell count continued to increase to a level of 3.24 million per cmm on the 27th day. Consideration of all clinical tests on AS and UFR fractions indicated that UFR fraction contained at least 6 times as much intrinsic factor as AS fraction per gram.

The degree of homogeneity of the AS and UFR fraction was investigated with the Tiselius electrophoresis apparatus. As indicated in

TABLE II. Clinical Tests on AS Fraction and a UFR Fraction Derived from It.*

Intrinsic factor preparation	Patient	Daily dose, mg	Red blood cell count (million/mm³) Initial	At 21 days	Reticulocyte peaks — Observed, %	Expected(1), %
AS fraction	S.M.	6.5	2.47	3.45	14.2 (8th day)	16
" "	K.K.	13.0	2.42	3.63	17.8 (9th day)	17
UFR fraction #21-2	E.K.	1.0 and 2.0†	2.25	2.67	7.3 (3rd day) on 1 mg/day 6.5 (15th day) on 2 "	19
UFR fraction #21-2	B.R.	2.0	2.04	3.68	20.1 (10th day)	22
UFR fraction #21-2	K.W.	2.0	2.44	3.86	18.6 (9th day)	16

* Tests were carried out by Dr. Tom Spies.

† This test carried out by Drs. Janet Watson and H. Lichtman. Dosage increased to 2 mg on 12th day and a second reticulocyte response resulted.

Fig. 1, AS fraction appeared quite heterogeneous when subjected to electrophoresis at pH 8.6 in barbiturate buffer or in acetate buffer at pH 4.2.

The marked increase in heterogeneity of AS fraction at pH 8.6 compared to pH 4.2 is probably due to decomposition of the material since zone electrophoresis experiments on paper have indicated that development of multiple components which occurred above pH 7.0 was coincident with a loss of intrinsic factor activity.

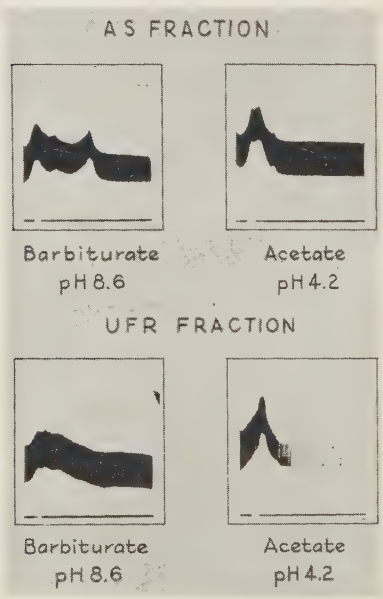

FIG. 1. Descending electrophoretic diagrams using Perkin-Elmer Model #38. Protein concentration of AS fraction = 0.67%; ionic strength = 0.1. Protein concentration of UFR fraction = 1.0%; ionic strength = 0.1. Time of exposure: upper left = 54 min.; upper right = 40 min.; lower left = 30 min.; lower right = 60 min.

UFR fraction No. 21-2 shown in Fig. 1 was less heterogeneous than AS fraction at either pH. The demonstration at pH 4.2 of one major component of UFR fraction together with the observed high clinical activity of this fraction suggested that this component was intrinsic factor or closely associated with it. UFR fraction behaved similarly to AS fraction in that conditions at pH 8.6 appeared to cause the development of multiple components. It was desirable to test homogeneity by an entirely different method, and the synthetic boundary cell for the Spinco ultracentrifuge, a cell which is especially suited for investigation of low molecular weight materials(4,5) was used. With this cell the initial boundary appears as a sharp peak in the middle of the diagram. As shown in Fig. 2, AS fraction at 32 minutes gave a broad asymmetric peak indicative of several components. The diagrams at 8 minutes reveal that UFR fraction contained high molecular weight material which sedimented rapidly showing a small peak to the right of the main peak. The remaining slow-moving, low molecular weight component gave sharp symmetrical peaks after 32 minutes of centrifuging in both acetate and barbiturate buffers, indicating good homogeneity.

As shown in Table III, intrinsic factor preparations showed an increase in glucosamine content during concentration. For maximum glucosamine values the extended hydrolysis method of Belcher *et al.*(6) was used.

From analysis and molecular weight calculation of the components shown in the UFR fraction diagrams in Fig. 2, Dr. R. A. Brown of these Laboratories pointed out that it would

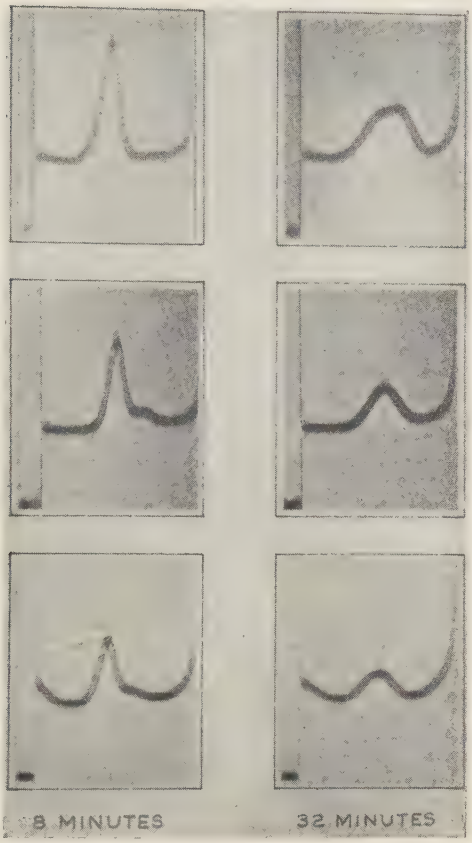

FIG. 2. Sedimentation diagrams using a Spinco Model E ultracentrifuge and a synthetic boundary cell. Top: AS fraction, acetate, pH 5.2. Center: UFR fraction, acetate, pH 4.3. Bottom: UFR fraction, barbiturate, pH 8.6. Speed = 59,780 r.p.m.; protein conc. = 0.6%; ionic strength = 0.15.

be possible to separate the low and high molecular weight components (LMW and HMW) contained in UFR fraction using the preparative head of the ultracentrifuge. Preliminary separation and ultracentrifuge analysis of both fractions showed that LMW component comprised about 70% of the protein. No heterogeneity could be detected in this component by the synthetic boundary cell and the molecular weight appeared to be about 5000. As yet incomplete clinical evaluation indicates that LMW, the major component, possessed the greater intrinsic factor activity. HMW was heterogeneous consisting of components with molecular weights of about 100,000 and about 500,000. Detailed physical, chemical and clinical studies of the separated components of UFR fraction are in progress and will be reported.

Zone electrophoresis studies on paper were carried out on AS and UFR fractions with the inverted V technic of Durrum(7). Using barbiturate buffer at pH 7.0 to pH 8.6 both fractions exhibited several components which separated poorly showing much overlapping. Preparative scale separation of UFR fraction at pH 7.0 yielded 5 subfractions all of which were clinically inactive. Since Latner *et al.* (8) had shown that active fractions could be separated at pH 6.35, we concluded that at pH 7.0 or above decomposition could occur with development of components of different charge and with loss of intrinsic factor activity. In our hands, electrophoresis of AS fraction at pH 6.1† revealed the presence of 4 major components moving toward the anode, and UFR fraction No. 21-2 showed 2 components both moving toward the anode at this pH.

Discussion. The clinical tests were at best semiquantitative since the amounts of the various fractions giving maximum response with *different* patients were compared, hence exact relative potencies could not be determined. The degree of purification of AS fraction represented an increase in intrinsic factor potency over that of hog stomach powder of about 150-fold and UFR fraction of about 1000-fold. Glass *et al.*(9,10) obtain a preparation described as being a single protein called "glandular mucoprotein" which was effective at a dose of 150 to 200 mg. Latner *et al.*(8,11) have obtained intrinsic factor preparations described as being homogeneous by paper electrophoresis and in the ultracentrifuge although but little information was given on the isolation and clinical evaluation of the preparation. Because of the high hexosamine content, which has now been confirmed by us, intrinsic factor was said to be a mucoprotein. Glass(12) questioned the originality of this claim pointing out his earlier description of the intrinsic factor activity of "glandular mucoprotein." The preparation of Glass *et al.* (9) appears to contain not more than 2%

† Michaelis buffer containing 3.885 g of sodium acetate, 5.885 g of sodium barbital and 280 ml of 0.1 N HCl/liter.

TABLE III. Nitrogen and Glucosamine Contents of Intrinsic Factor Preparations.

Material	Nitrogen content, %	Glucosamine content, %
Hog stomach powder	12.2	2.54
AS fraction	12.6	5.85
UFR fraction, #21-2	11.8	15.2

intrinsic factor since 200 mg were needed to produce an adequate response. Obviously the chemical properties of this crude preparation do not necessarily reflect the properties of intrinsic factor. Our fraction of molecular weight about 5000 perhaps is more properly called a mucopolypeptide. Prusoff *et al.*(2,13) described preparations effective at 15 mg and Welch and Heinle(14) briefly referred to a preparation from human gastric juice effective at 0.6 mg daily in one patient. With the exception of this last report, UFR fraction at a dose of 2 mg daily is the most potent intrinsic factor preparation reported to be effective in therapy of pernicious anemia patients in relapse. Latner *et al.*(9) have found that 1 and 2 mg of their best preparations will cause detectable intestinal uptake of radioactive vitamin B_{12} according to the fecal excretion technic of Welch *et al.*(15). The quantitative relationship between activity in this test and response of pernicious anemia patients in relapse has not been established.

Latner *et al.*(8) reported the molecular weight of the major component in their preparation as being about 15,000 to 20,000. The lower molecular weight of our LMW fraction may possibly be due to the trypsin and chymotrypsin treatment. Ultracentrifugal analysis at pH 8.6 did not reveal the presence of components of different molecular weight. This suggests that the decomposition at pH 8.6 was a change in charge resulting from minor changes in the molecule rather than from changes in molecular weight. Prusoff *et al.*(2) discussed the status of vit. B_{12} binding as an assay for intrinsic factor and showed that this technic is non-specific. The low vit. B_{12} binding capacity of UFR fraction of 220 γ/g of fraction further supports their conclusion. Ascribing all the binding capacity of UFR fraction to the low molecular weight component would require that over 1000 molecules of this component be necessary to bind 1 molecule of vit. B_{12}. It appears that vit. B_{12} binding is not a property of intrinsic factor.

Summary. A highly potent intrinsic factor preparation has been obtained from desiccated hog stomach by ammonium sulfate fractionation, digestion with proteolytic enzymes, alcohol fractionation and finally ultrafiltration. The ultrafiltration residue fraction proved effective in pernicious anemia patients in relapse at a daily dose of 1 or 2 mg together with vit. B_{12}. Electrophoresis and ultracentrifuge studies of this fraction showed that the major portion of the material was a component with a molecular weight of about 5000. Preliminary separation in ultracentrifuge and clinical evaluation of this homogeneous component suggested strongly that it was intrinsic factor. The ultrafiltration residue fraction contained 15.2% glucosamine and 11.8% nitrogen. In confirmation of our analysis for hexosamine, UFR fraction No. 21-2 was found by Dr. Saul Roseman, University of Michigan, Ann Arbor, to contain 14% hexosamine HCl. By a new differential colorimetric method developed by Dr. Roseman about half of the hexosamine was glucosamine HCl while the remainder was presumably chondrosamine HCl.

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Potassium and Reducing Substances in Proximal Tubule Fluid of the Rat Kidney.* (21395)

HEINRICH WIRZ† AND PHYLLIS A. BOTT. (Introduced by A. N. Richards.)

From the Department of Physiological Chemistry, Woman's Medical College of Pennsylvania, Philadelphia.

It has been reported(1,2) that preliminary results on tubule fluid collected from the kidney of Necturus indicate reabsorption of potassium by the proximal tubule, while inulin is being concentrated. In mammals no direct proof for reabsorption of potassium by the proximal convoluted tubules has been established so far, but it has been computed by indirect methods(3,4). In reviving the micropuncture technic for the study of mammalian kidneys, we believed potassium concentration to be of sufficient interest to investigate it first. Furthermore in Necturus the decrease in potassium over the region where glucose has been shown to be reabsorbed from the amphibian kidney tubule fluid(5) and mammalian tubule fluid(6) suggested interdependence of the reabsorption mechanisms for these two substances.‡ For this reason the determination of reducing substances was included when possible.

Methods. Large male albino rats were used. Methods of anesthesia, preparation for visualization of kidney surface, procedures of micropuncture and collection of tubule fluid as well as identification of site of puncture have been depicted in detail by Walker and Oliver(7), and nothing is to be added except a few minor modifications: Unilateral nephrectomy was performed in only 2 cases (one kidney had been removed 12 days previously for Exp. 4 and 13 days previously for Exp. 5 of Table I). It is felt however that unilateral nephrectomy offers great advantage not only because of the ensuing hypertrophy of remaining kidney but chiefly because of its compensatory hyperfunction. Usually intestines from upper jejunum to transverse colon were excised after mass ligature. This procedure, besides being very easily achieved has the advantage of leaving the arterial blood supply to the liver intact. Yet the removal of a large part of the intestinal tract creates enough room for controlling properly the respiratory movements of the kidney by one or two glass rods. The left ureter was cannulated for collection of urine. Mild *osmotic diuresis* was induced by intravenous injection of mannitol. Usually 2 or 3 ml, rarely 1 ml of a 5% solution were sufficient to secure a moderate diuresis which lasted through the collection time. The procedure of identifying *site of puncture* by microdissection of macerated kidney was simplified somewhat. As stated by Walker and Oliver the most cumbersome part of the manipulation is the disentanglement of the distal convolution from surrounding coils of proximal one. For this reason we usually did not attempt to separate and measure the whole nephron but only the proximal convolution. The isolated proximal tubule was transferred to a glass slide; its total length and the distance to site of puncture measured—section by section—by means of an eye piece micrometer. *Blood samples* for reducing substances were taken from the ani-

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† Swiss National Research Foundation Fellow. Present address: Physiological Institute, University of Basle, Switzerland.

‡ Mudge *et al.*(3) attempted to show a reduction of renal tubular reabsorption of potassium in addition to glucose by the use of phlorhizin in indirect experiments in dogs but their results were negative.

TABLE I. Results of Analyses of Proximal Tubule Fluid.

No.	Man., ml	Collection			Potassium				Glucose			Urine
		Site*	Amount, μl	Rate, μl/hr	Fl. mEq/l	P.	Fl./P.	U./P.	Fl. mg/100 ml	P.	Fl./P.	Rate, ml/hr
1	2	3.9/11.8	.58	.39	5.56	6.47	.86	.70	182	255	.71	.25
2	3	4.5/13.4	.35	.66	4.40	5.51	.80	4.14	110	364	.30	.30
3	2	4.1/10.1	.22	.12	4.08	7.06	.58	1.77	—	—	—	.06
4†	1	5.2/11.7	.41	.41	2.12	4.92	.43	19.00	136	275	.49	.40
5†	2	5.3/11.6	.50	.50	2.05	2.76	.74	20.30	266	348	.76	.60
6	3	4.3/ 9.4	.25	.15	3.64	4.34	.84	1.13	—	—	—	.25
7	3	4.8/10.1	.15	.10	1.48	5.74	.26	1.66	—	—	—	.17
8	2	6.0/11.9	.11	.08	3.44	6.05	.57	<.10	—	—	—	.10
9	2	5.7/ 9.9	.57	.36	5.48	4.82	1.14	4.18	198	279	.71	.19

Man. = 5% mannitol injected intrav.; Fl. = tubule fluid; P. = blood plasma (a correction of 7% has been added to account for plasma proteins); U. = ureteral urine.

* Numerator of the fractions in this column is the measured distance, in ml, from the beginning of the convolution to the point of the fluid collection; denominator is measured total length of the convolution.

† Unilaterally nephrectomized animals.

mal's tail before and after collection of tubule fluid. All blood samples were centrifuged immediately for 2 minutes in pyrex capillary tubes which had been heparinized. Average was taken by graphic interpolation over collection time. Potassium was determined either in the same capillary plasma or in plasma of carotid blood taken at end of the experiment.

All *plasma values* were multiplied by a factor of 1.07 to account for plasma proteins. *Potassium* was determined in urine by use of internal standard flame photometer as described by Fox(8). In tubule fluid and plasma it was determined by an ultramicro method developed by one of us(9) which will be described in detail elsewhere. When applied to fresh rat plasma it yielded results within 0.2 mEq/l of those obtained by the macro flame photometer method. *Reducing substances* were estimated in plasma and tubule fluid by ultramicro adaptation of the Sumner method as described by Walker and Reisinger(10). Colorimetry was done with an Evelyn colorimeter, modified as follows: One opening of the carriage of the micro set was covered by a brass plate bearing a slit of 0.3 mm in width and 6 mm in length. The pyrex tubes, outer diameter 0.5 mm, containing the colored solution, were fixed to this slit by means of a spring clamp. The slit with a tube attached to it was centered so that a maximum of light reached the photo cell, and the whole unit firmly fastened in this position to the carriage. A galvanometer of sensitivity 0.0006 μAmp/mm, and a No. 515 filter were used. With galvanometer set at 100 by means of a blank, an almost straight standard line results with glucose concentrations up to 500 mg/100 ml if plotted on semilogarithmic paper. Time is gained with the photoelectric modification without sacrifice of accuracy.

Results. As no special procedure was employed to identify the tubules *in vivo* and fluid was collected from any random superficial segment it was disappointing though not surprising that all punctures happened to be located in the middle third of the proximal convoluted tubule. The 9 experiments summarized in Table I show that with one exception the potassium concentrations are definitely lower in tubule fluid than in plasma.§ This indicates active potassium reabsorption by the proximal convoluted tubules in the rat as in Necturus (1,2). Since in mammals considerable reabsorption of water has been observed in this same region by Walker, Bott, Oliver and MacDowell(6) the active potassium reabsorption becomes even more conspicuous. It is not possible however to make a definite statement as to the degree of potassium removal from tubular fluid as the amount does not appear to be uniform. In the single experiment (Exp. 9) in which the potassium fluid/plasma ratio was greater than 1 there must still have been con-

§ In one additional experiment not reported in the table because of the possibility of certain flaws there is no doubt that the tubule fluid potassium is lower than that of plasma.

siderable reabsorption of potassium if water reabsorption was normal for this region. On the other hand it is possible that the reabsorbing mechanisms for potassium and glucose were both unusually inactive in this tubule. The animal appeared to be normal and a qualitative test for sugar on the urine gave a negative result.

In one instance (Exp. 7) a tubule fluid/plasma ratio of as little as 0.26 has been stated. If potassium reabsorption continues along the inaccessible last third of this segment, potassium might be removed completely or nearly completely from tubular content. Mudge, Ames, Foulks and Gilman(3) and Berliner, Kennedy and Orloff(4) have concluded from indirect evidence in dogs that a large proportion if not all of the urinary potassium might be attributed to tubular secretion possibly by some distal part of the nephron. This assumption implies the necessity for a complete or nearly complete potassium reabsorption at a more proximal level. In general the results of these micropuncture experiments demonstrate the possibility of this suggestion as they disclose a proximal potassium reabsorption which might well become complete by the end of the proximal convolution. If it does become complete the urine potassium figures of most of our experiments would have to be explained by distal tubule secretion of potassium. However, without actual figures for fluid from the end of the proximal tubule we must permit another explanation of these results: an incomplete proximal potassium reabsorption and a concentrating effect of distal water removal. The strikingly higher U/P ratios in the unilaterally nephrectomized animals may carry a suggestion of increased secretion in these cases.

Reducing substances were determined whenever the amount of fluid collected from a tubule was sufficient. The 5 results are summarized in the Table. Compared with the results of Walker, Bott, Oliver and MacDowell the tubule fluid/plasma ratios for reducing substances are somewhat larger. This is obviously due to the fact that plasma glucose levels in our experiments were consistently higher than theirs. Whereas the findings of Walker *et al.* led to the conclusion that glucose reabsorption increased with a moderately increasing load, it appears here to approach a threshold at very high plasma levels. The reason for the hyperglycemia observed in this series is obscure. It was noted that plasma glucose was within normal limits at the beginning of the experiments and tended to increase as time proceeded.

It is evident that glucose reabsorption occurs along the same region as that of potassium. An interconnection of the two reabsorptive processes has not been clearly demonstrated in the five experiments in which these could be studied as it seems possible for the tubule fluid/plasma ratios of the two compounds to vary independently. The approach to identity in three of the cases, however, warrants further investigation of this point.

Summary. The site of renal tubular potassium reabsorption has been investigated by micropuncture studies in rats. In 8 out of 9 experiments potassium concentrations in fluid collected from the middle third of the proximal convolution are lower than in plasma. Potassium therefore appears to be reabsorbed actively by the proximal convoluted tubules. The site of this process is roughly identical with that of active glucose reabsorption.

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Impairment of Spermatogenesis in the Rat After Cutaneous Injection of Testicular Suspension with Complete Adjuvants.* (21396)

Jules Freund, Murray M. Lipton, and George E. Thompson.

From the Division of Applied Immunology, Public Health Research Institute of The City of New York.

It has been shown that the guinea pig develops aspermatogenesis following a single intracutaneous injection of spermia or testicular suspension incorporated into a water-in-paraffin oil emulsion containing killed mycobacteria(1).† The aspermatogenesis is characterized by the degeneration and necrobiosis of germinal cells with no injury to the Leydig cells or changes in the seminal vesicles and prostate. When the autologous or homologous testicular material is replaced by a similar suspension from other organs of the guinea pig or by testicular material from other animals, such as bull, sheep, or rabbit, aspermatogenesis does not develop in the guinea pig. Thus, this process appears to be organ and species-specific. It is noteworthy that repeated injections of the homologous testicular material in salt solution or in a water-in-paraffin oil emulsion without mycobacteria fail to produce aspermatogenesis; similarly, the injections of (a) testicular material in water-in-oil emulsion and (b) killed mycobacteria in paraffin oil into 2 separate sites do not induce testicular injury. Because of the specificity of the process and the effect of the adjuvants, it may be assumed that the aspermatogenesis is the result of sensitization of immunological nature.

The purpose of the present experiment was to determine if aspermatogenesis can be produced by similar means in the rat. This species was selected because, in contrast to the guinea pig, it is not readily sensitized. In addition, it is the species of choice for endocrinological and nutritional studies.

Methods. Rats of Wistar or Sherman strain, most of them weighing from 150 to 250 g, were fed on Rockland rabbit chow and given water *ad libitum*. They were injected into the skin of the nuchal and dorsal‡ regions (above the lumbar region) with 7 simultaneous injections of 0.1 ml each of an emulsion containing a suspension of rat testis in 0.85% salt solution combined with paraffin oil and killed tubercle bacilli. Each 0.1 ml contained 25 mg (wet weight) testis, 0.025 ml of 0.9% NaCl solution, 0.0075 ml Arlacel A (emulsifying agent), 0.04 ml paraffin oil, and 0.029 mg (dry weight) killed tubercle bacilli. For details of the technic see (1). At the intervals of time indicated in Table I the injections were repeated. In control experiments, rats of the same strain and weight were given

TABLE I. Impairment of Spermatogenesis in Rats after Intracutaneous Injections of Homologous Testicular Suspension Combined with Complete Adjuvants. (Control rats with homologous liver or kidney suspension instead of testicular material.)

Exp.	Schedule of inj., days of exp.	Killed after last inj., days	Degree of injury*		
1	1, 14, 32	15		±	3+
		50		4+	4+
		57			3+
2	1, 8, 14	9	0	0	1+
		15			0
	1, 8, 14, 44	7			1+
		19	0	±	2+
					4+
		26 to 29	0	0	0
			0	0	0
			±	1+	1+
				2+	3+
3	1, 8	50		1+	2+
	1, 8, 39, 69	8			1+
		20			3+
4 Controls	1, 8, 20, 50	18 to 21	All 24 rats: 0		

Controls: 12 rats with liver, 12 rats with kidney suspensions.

* Each figure refers to one rat.

* Supported in part by a grant-in-aid from the American Cancer Society upon recommendation of the Committee on Growth of the National Research Council.

† Complete adjuvants = Emulsifying agent such as Arlacel A or Falba or Aquaphor and paraffin oil containing killed mycobacteria.

‡ It is essential to avoid injection sites near the testes.

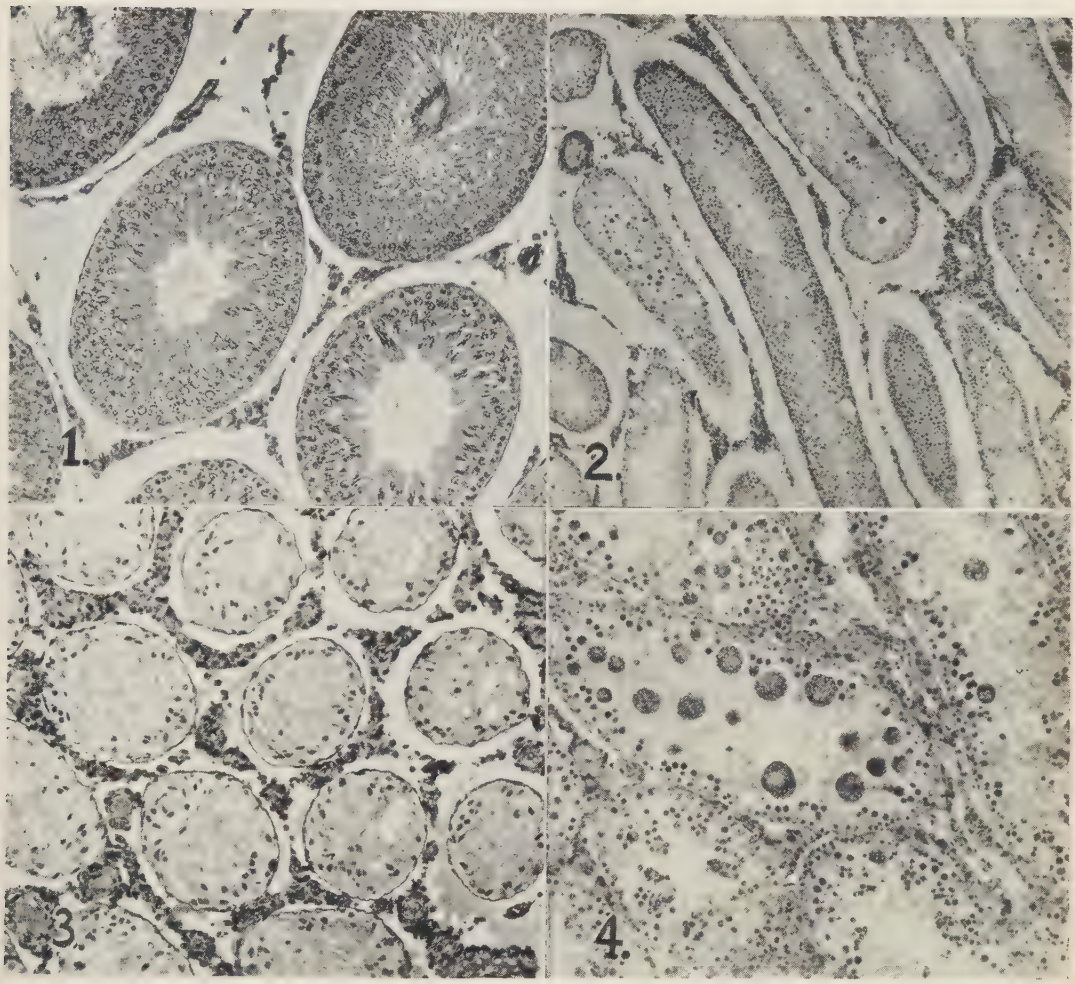

FIG. 1. Normal testis, × 130. FIG. 2. Testis, 2+ damage, × 66. FIG. 3. Testis, 4+ damage, × 130. FIG. 4. Testis, 4+ damage, multinucleated cells, × 130.

the same kind of injections, but the testicular material was replaced by suspensions of rat liver or kidney. The rats were killed at stated intervals after the injections by exsanguination under chloroform anesthesia. The testes, seminal vesicles, and the prostate were fixed in Bouin's solution, while the sites of injections, lungs, spleen, liver and kidney were preserved in 10% formalin.

In a proportion of rats, impairment of spermatogenesis was found, and damage was graded as follows:

0, No damage.

±, Diminished number of spermia in seminiferous tubules and in epididymis; a few degenerated and exfoliated germinal cells.

1+, Very few mature spermia; a fairly large number of degenerated germinal cells, some being exfoliated and present in epididymis

2+, Most seminiferous tubules lacking mature spermia; large number of degenerated germinal cells, many necrobiosed and exfoliated.

3+, Mature spermia absent, most of the seminiferous tubules containing only 1 or 2 layers of germinal cells; degeneration, pyknosis, exfoliation conspicuous, some tubules devastated, others containing wavy acidophilic precipitates.

4+, Injury ranging from partial to com-

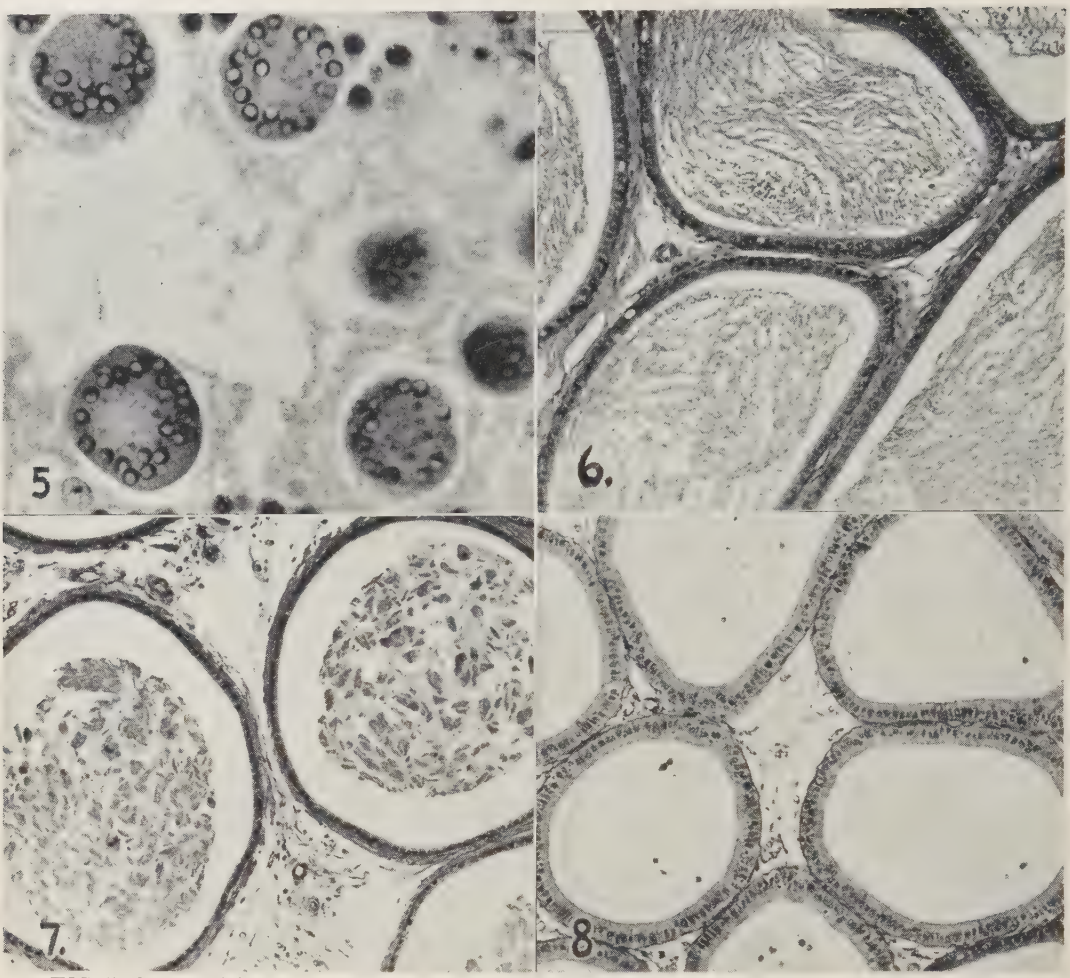

FIG. 5. Same as Fig. 4, × 500. FIG. 6. Normal epididymis, × 130. FIG. 7. Epididymis with mucoid and exfoliated, dead germinal cells, × 130. FIG. 8. Epididymis, total absence of spermia, × 130.

plete devastation of the tubules with only Sertoli cells and basement membrane remaining. Frequently multinucleated large round cells with dark staining, round nuclei. Focal accumulation of large mononuclear cells and lymphocytes about some tubules.

Testis and epididymis with and without injury are shown in Fig. 1-8.

The results of representative experiments are given in Table I which shows that in a considerable portion of the rats impairment of spermatogenesis was induced by the repeated injections of homologous testicular suspension combined with paraffin oil and killed mycobacteria. In additional experiments impairment of spermatogenesis was induced in several groups of rats whose treatment differed from that reported only with regards to the intervals of time between injections. When the testicular suspension was replaced by suspensions of rat liver or kidney, repeated injections failed to cause impairment of spermatogenesis.

The seminal vesicles and prostate were normal in size and there were no microscopic changes in the secreting cells. The secretion was abundant.

At the sites of injections of organ suspension plus paraffin oil containing killed tubercle bacilli, granulomatous lesions were found, as described in a previous paper on the production of allergic encephalo-myelitis in the rat

(3). Similar reactions were present in some of the regional lymph nodes.

The lungs of some of the rats, both in the experimental and control groups revealed focal accumulation of large mononuclear cells with microscopic oil vacuoles, and lymphocytes.

The livers, kidneys and spleens of the rats were normal. The lungs of some rats were not free of mild bronchitis. However, a few of the rats developed intercurrent diseases with moderately advanced bronchitis and interstitial pneumonia. These animals were excluded from the report. Since intercurrent diseases or nutritional factors may lead to impairment of spermatogenesis, it is of interest that no testicular damage was found in rats, which in concurrent experiments, developed allergic encephalo-myelitis following the intracutaneous injection of suspensions of spinal cord combined with complete adjuvants.

Discussion. The experiments just described show that 19 of 29 rats developed a varying degree of aspermatogenesis following repeated intracutaneous injections of testicular suspensions combined with adjuvants. These lesions did not occur when liver or kidney suspensions were substituted for the testicular material. The lesions were not associated with inflammation in the intertubular space except in some far-advanced cases. The Leydig cells, the seminal vesicles, and prostate remained normal; thus the aspermatogenesis is not likely to be caused by interference with the production of sex hormones.

The multinucleated cells found in the seminiferous tubules in some of the rats with severe testicular injury are rarely seen in impairment of spermatogenesis due to other causes. They were found, however, in Mason's(2) classic experiments on aspermatogenesis in rats kept on vit. E deficient diet.

A comparison of the impairment of spermatogenesis induced in the rat and the guinea pig(1) shows that, under the conditions of the experiments, the rat is less susceptible to the effect of the injection of homologous testicular material and adjuvants than the guinea pig. Even after repeated injections, only a little more than half of the rats developed aspermatogenesis of varying degree, while after a single injection, all of the guinea pigs had severe impairment of spermatogenesis. It is noteworthy that in the rats killed 50 and 57 days after the last injection of Exp. 1, $3\pm$ or $4\pm$ injury was present, with no indication of recovery. The immunological aspect of these observations was discussed in previous papers(1,3,4).

Summary. (1) Impairment of spermatogenesis was induced in 2 out of 3 albino rats by repeated injections of a suspension of rat testis combined with paraffin oil and killed mycobacteria. (2) The injury selects the germinal cells; the Leydig cells, seminal vesicles and prostate remain unaffected. (3) When severe injury occurs, large multinucleated cells may appear in the seminiferous tubules.

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Effect of pH on Platelets and Identification of Ribonucleoprotein from Platelets.* (21397)

Richard W. Greene. (Introduced by E. L. Lozner.)
(With the technical assistance of Rosemarie Blanchard.)
From the Department of Medicine, State University of New York, Syracuse, N. Y.

Schulte(1) first described the agglutination of platelets in isotonic salt solution at pH 3.5 to 4.5. Later Horowitz(2) stated that the "smallest changes in pH produce agglutination of platelets." Agglutination to these workers implied the process of viscous metamorphosis as originally proposed by Wright and Minot (3), and not an antigen-antibody reaction. Moderate change in pH did not affect the clot retraction ability of platelets according to Ellicott and Conley(4). In a recent patient with chronic idiopathic thrombocytopenic purpura(5) exhibiting a high titer of a circulating platelet agglutinin, it was found that varying the pH from 5 to 9 affected minimally the platelet agglutinating titer of the serum.

In the investigation by others concerning the origin of platelets and the elucidation of their structure, it was discovered that they did not contain nuclear material (*i.e.* desoxyribonucleic acid) because they produced a negative Feulgen's reaction(6). More recently Wagner(7) reported that platelets contained rather large quantities of ribonucleic acid (RNA) and that all the nucleic acid content of platelets was of the ribose type. In the present study, the effect of changes of pH on whole washed platelets was reinvestigated and efforts were made to determine the major components of platelets.

Experiments. Platelets were prepared from several 50cc specimens of human blood according to the methods of Dillard, Brecher and Cronkite(8), except that following the centrifuging of platelets from plasma, the platelets were washed twice with 0.15 M sodium chloride, and then the samples were pooled. When strict attention was paid to the speed and time of the centrifugations, platelet preparations containing less than one red cell per 2000 platelets and less than one white cell per 5000 platelets were easily obtained. Samples of a single platelet preparation were resuspended in acetate or phosphate buffers of varying pH. The ionic strength was constant at 0.15.

Results. As the pH decreased from 7 to 5, platelet clumping increased—judged by microscopic examination and by the rate that the platelets settled. Below pH 5 there was marked clumping. If the pH was lowered from 4.5 to 3.0, there appeared to be no further clumping. If the pH was lowered to 4.5 and then raised to 7.5, the clumps decreased in size by gross inspection. However, microscopically there were numerous clumps of from 10 to 20 platelets which persisted even after vigorous shaking. But if the platelets were unexposed to a pH of 4.5, they remained separate and discrete elements. Between pH 7.0 and 10.0, the platelets stayed suspended even after several hours. Between pH 10.0 and 10.5, the platelets dissolved completely. Fig. 1 illustrates these phenomena.

Further investigation involved the platelets which had been dissolved at pH 10.5. This step was performed at 0° to 5°C. After obtaining the solution of the platelets, siliconed glassware was used no longer. The concen-

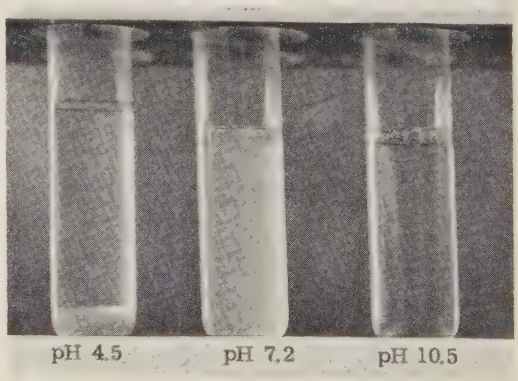

FIG. 1. Photograph taken 2 hr after mixing platelets and buffer.

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trated solution was very viscous. Apparently gross denaturation began within 6 to 8 hours when part of the solution began to form a gel-like mass. This evidence of denaturation was much more rapid at pH 10.5 than at pH 7.5 and also more rapid at room temperature than at 0° to 5°C. The following characteristics were observed on the fresh preparation. It contained 13.4% nitrogen, 0.98% phosphorus. In ultraviolet light, it had a minimum absorption at 254 mu and a maximum absorption at 272 mu with an E (1%, 1 cm) of 14.9 at this latter wave length. It precipitated if dialyzed against distilled water—resolution easily taking place by the addition of small amounts of sodium chloride. Raising the ionic strength to 5.0 had no visible effect. The native fresh material gave a strong positive reaction for ribose(9)† using Bial's test, and a negative Dische test for desoxyribose using diphenylamine. Approximately 10^{10} platelets produced 11.2 mg of the material. Assuming platelets have an average volume of 7.5 cu. u(9), the solid material concentration inside platelets was calculated to be 15%, a reasonable value for cytoplasmic substances(10).

Electrophoresis was performed at pH 7.37, ionic strength 0.15, phosphate buffer; and at pH 8.6, ionic strength 0.1, barbiturate buffer. In the preparation of the protein for electrophoresis by dialysis, a small amount of the gel-like denaturated protein was centrifuged off. Therefore, possibly the electrophoresis did not represent the only substance inside of platelets. However, it is hypothesized that the electrophoresis represented the great majority of platelet substance and that the gel-like precipitate which was centrifuged out was the denatured product of the same substance which was analyzed. Both at pH 7.37 and 8.60 the material moved essentially as a single boundary. Approximate mobilities were as follows: at pH 7.37, 4.4 x 10^{-5} cm^2/volt sec.; at pH 8.60, 3.5 x 10^{-5} cm^2/volt sec. These mobilities are slightly lower than ribonucleoprotein (RNP) from liver(11).

If the pH of the native fresh material were lowered below 6.8, a flocculent precipitate formed. This was centrifuged. The supernatant gave a strongly positive test for ribose and had a sharp absorption peak at 262 mu. The precipitate did not dissolve at pH 7.5, but did dissolve at pH 10.6. This dissolved precipitate gave a negative ribose test and had a broad ultraviolet absorption peak—maximal at 282 mu.

Discussion. It was concluded that inside of platelets there was one major substance—ribonucleoprotein (RNP). This RNP denatured rather easily. This rapid denaturation may be inherent in the molecule and/or due to the presence of ribonuclease. At acid pH the RNA was split from the RNP. The most accepted theory as to the origin of the platelets is that they are cytoplasmic fragments of megakaryocytes. Therefore, the finding that the major substance of platelets is RNP is in agreement with this theory since in general there are large quantities of RNP in the cytoplasm of cells(12). Nucleoproteins are large molecules consisting of proteins, nucleic acids, and lipids(12). Hence, this report does not disagree with other investigators who have analyzed platelets for protein, fat, etc. (13,14); nor does it disagree with Zucker, *et al.*(15) who found small quantities of serotonin in platelets, as this investigation was not concerned with trace substances. Furthermore, the separation of two substances, the granulomere and the hyalomere, from platelets by Fonio(16), and the various "platelet factors" summarized by Stefanini(17) which affect blood coagulation, may be due to a partial denaturation of the ribonucleoprotein complex—the different components of this complex having different physiological functions.

Summary. 1. Platelets agglutinated at acid pH, maximal at pH 4.5. They dissolved at pH 10.5. 2. The major substance of platelets was found to be ribonucleoprotein.

† Bial's test is not specific for ribose but is a generalized test for pentose sugars.

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Conversion of 15th Carbon of Palmitic Acid to CO_2 by the Intact Rat.* (21398)

E. O. WEINMAN,† W. J. LOSSOW, I. L. CHAIKOFF, AND W. G. DAUBEN.

From Department of Physiology, School of Medicine, and Department of Chemistry, University of California, Berkeley.

In an earlier investigation we studied the relative rates at which the carboxyl, 6th and 11th carbon atoms of palmitic acid are converted to CO_2 by the intact rat(1). It was observed that the location of the C^{14} in the palmitic acid chain did not influence significantly the amounts of $C^{14}O_2$ recovered. It was therefore concluded that, once the process of breakdown of palmitic acid is initiated in the intact animal, a palmitic acid molecule is disrupted in such a manner that all of its carbons are converted to CO_2 at about the same time.

In the meantime, studies with liver slices and homogenates revealed that the fate of the terminal 2-carbons of the methyl end of a fatty acid differs considerably from that of its other carbons. This was observed for octanoic acid by Crandall *et al.*(2) and for palmitic acid, in this laboratory, as described by Brown *et al.*(3). It next seemed necessary to compare, *in the intact animal,* the over-all conversion to CO_2 of the 15th carbon of palmitic acid with that of its other carbons.

Experimental. Preparation of Emulsions Containing Tripalmitin-15-C^{14}. The synthesis of tripalmitin-15-C^{14} has been described by Brown *et al.*(3). Emulsions of this labeled triglyceride were prepared exactly as described in our previous publication(1). The emulsions contained, by weight, 2% tripalmitin, 2% olive oil, 1% glycerolmonostearate, 5% glucose, and 90% water. The average diameter of visible particles approximated 1 μ and thus corresponded to Exp. II in our previous report(1). *Treatment of Animals and Collection of CO_2.* The experimental conditions of this investigation were also identical with those of our previous study. Female rats of the Long-Evans strain, weighing about 170-180 g were used. Rats which had been fasted for 24 hours were lightly anesthetized with ether and then injected, via the foot vein, with 1 ml of the tripalmitin-15-C^{14} emulsion. Immediately thereafter the rats were placed in glass cages ventilated with CO_2-free air. The expired CO_2 was absorbed in towers containing carbonate-free sodium hydroxide solutions, and was collected continuously for 24 hours. During that period, 16 separate samples were obtained at various intervals, the shortest be-

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† Present address: Department of Chemistry, University of California, Los Angeles.

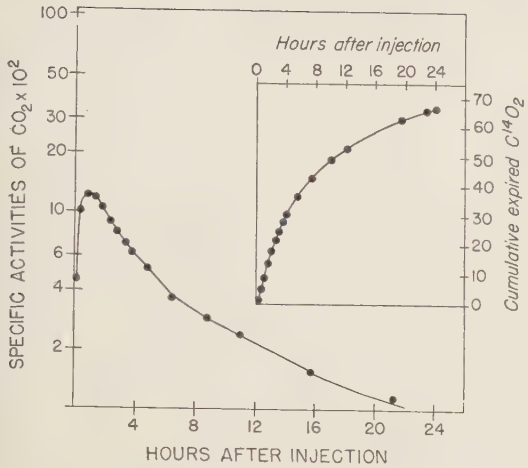

FIG. 1. Each curve represents avg obtained from 5 rats. Semilogarithmic plot of *specific activities* of expired CO_2 against time. Specific activity refers to the % of injected palmitic acid-C^{14} recovered in expired air/mg of CO_2-carbon. *Cumulative percentages* of injected palmitic acid-C^{14} recovered in expired CO_2.

ing 20 minutes. During the experiments the rats had access to water, but food was withheld. *Analysis of Expired CO_2.* The methods used for determination of CO_2 and $C^{14}O_2$ were those described by Entenman *et al.*(4).

Results. The specific activity-time relationships of the expired CO_2 and the cumulative percentages of injected C^{14} recovered in the expired air are shown in Fig. 1. These curves represent the average values obtained from 5 rats. In 24 hours, an average of 66% of the injected C^{14} was expired as CO_2, and the individual values ranged from 61 to 73%. These values agree closely with the cumulative (24-hour) values obtained previously with corresponding emulsions containing the C^{14} label on either the carboxyl, 6th or 11th carbon of palmitic acid (Exp. II(1)). In Exp. II of our previous investigation, the cumulative (24-hour) values ranged from 59 to 69%.

The specific activity-time curves of the expired CO_2 obtained here with tripalmitin-15-C^{14} also resemble closely the general shape of the specific activity-time curves derived from either tripalmitin-1-C^{14}, tripalmitin-6-C^{14}, or tripalmitin-11-C^{14} (compare with Fig. 2 in (1)). Maximum specific activities were again observed about one hour after injection of the labeled tripalmitin.

Thus it appears that, once the breakdown of a palmitic acid molecule is initiated in the intact rat, the entire molecule is disrupted very rapidly and *all* its carbon atoms become converted to CO_2 at practically identical rates.

Discussion. Lynen(5) has suggested that all C_2 units of a fatty acid chain form acetyl-S-CoA directly except for the C_2 unit derived from the methyl end, which at first yields acetyl-enzyme. This "acetyl-enzyme fragment"‡ may recondense directly with an acetyl-S-CoA unit (then constituting the carbonyl moiety of the resulting acetoacetyl-S-CoA molecule) or may be transformed to acetyl-S-CoA according to this reaction

$$CH_3\text{-}CO\text{-}Enzyme + HS\text{-}CoA \rightleftharpoons CH_3\text{-}CO\text{-}S\text{-}CoA + Enzyme \qquad \text{Eq. (1)}$$

This hypothesis readily explains the observations, by Crandall *et al.*(2) and Brown *et al.* (3), with rat liver slices, that the terminal two carbons distal to the carboxyl group of a fatty acid, (in contrast to other carbon pairs) preferentially enter the carbonyl moiety of acetoacetate. Brown *et al.*(3) also showed (as might be expected from this hypothesis) that, in *rat liver slices,* less $C^{14}O_2$ is produced from tripalmitin-15-C^{14} than from tripalmitin-1, -3, -5, -11, and -13-C^{14}. Presumably the 2-carbon fragment derived from the terminal carbon pair (*i.e.,* Lynen's acetyl-enzyme) is converted to acetyl-S-CoA according to Equation 1 before it condenses with oxalacetate (3). In our studies with the *intact rat,* however, no measurable difference was found in the rate of $C^{14}O_2$ production from tripalmitin labeled with C^{14} in either the 1st, 6th, 11th, or 15th carbon. In this connection Lorber *et al.* (6) noted (also with fasted, intact rats) that, even though more of the carboxyl than of the 7th carbon of octanoic acid was incorporated into liver glycogen, these two carbon atoms formed identical amounts of CO_2.

The liver glycogen findings of Lorber *et al.* (6) and the CO_2 findings, with liver slices, of Brown *et al.*(3) may be explicable as hepatic phenomena. (In each case there was greater incorporation from the nonterminal than from

‡ Beinert and Stansly(14) suggest that the enzyme-bound acetyl group is in the form of Enzyme-acetyl-S-CoA.

the terminal carbon pair of the fatty acid.) Our CO_2 findings, as well as those of Lorber and coworkers (equal CO_2 formation from both types of carbon pairs by the *intact* rat), represent, on the other hand, a balance of hepatic and extrahepatic processes.

In the intact animal, formation of CO_2 from 2-carbon fragments derived from the oxidation of palmitic acid may occur either "directly," when 2-carbon fragments condense with oxalacetate to enter the tricarboxylic acid cycle, or "indirectly" via acetoacetate which, for the most part, is formed in the liver and oxidized in extrahepatic tissues(7,8). If 2-carbon fragments derived from the terminal 2-carbons of a fatty acid enter the tricarboxylic acid cycle less readily than do the 2-carbon fragments derived from other carbon pairs, a larger fraction of the former may appear in acetoacetate. It is therefore possible that, under a given set of conditions,§ a larger proportion of two-carbon fragments derived from the terminal 2 carbons than of those derived from the other carbons of a fatty acid follows the "indirect" pathway of CO_2 production. In this event our data would indicate that, in the intact rat, at least under our experimental conditions, CO_2 is formed equally rapidly by either the "direct" or "indirect" pathway.

It may be argued here that, since the breakdown of free acetoacetate also proceeds by fragmentation to 2-carbon units, such fragmentation of an acetoacetate molecule should also give rise to the 2 types of 2-carbon fragments which could again show different reactivities toward oxalacetate. However, since the ability of extrahepatic tissues to oxidize acetoacetate exceeds by far their ability to form it, since lipogenesis is severely curtailed in fasted animals(9-12), and since other acetylating reactions may be considered to be of minor importance under our conditions, it would seem that, under such conditions, in extrahepatic tissues, the "terminal fragment" (*i.e.*, Lynen's acetyl-enzyme unit) is readily converted to acetyl-S-CoA which then condenses with oxalacetate. The findings of Chapman *et al.*(13) are of interest in this connection for these workers have reported that, with kidney slices, approximately equal percentages of $C^{14}O_2$ were recovered from octanoic acid-1-C^{14} and -7-C^{14}, whereas with liver slices, much less of the carboxyl than of the 7th carbon was incorporated into the CO_2.

Summary. 1. Palmitic acid labeled with C^{14} in its 15th carbon atom was injected intravenously, in the form of its triglyceride, into fasted rats, and the expired CO_2 was collected at various time intervals. 2. The amounts of $C^{14}O_2$ expired and the specific activity-time relationships of the expired CO_2 after the injection of tripalmitin-15-C^{14} correspond closely to the results reported previously with tripalmitin-1-C^{14}, tripalmitin-6-C^{14}, and tripalmitin-11-C^{14}. Our present findings thus support our previous postulate that the fasted *intact rat,* in contrast to certain isolated tissue preparations, converts all carbon atoms of palmitic acid to CO_2 at practically identical rates. 3. Possible explanations which may account for the differences observed between intact animals and isolated liver preparations are examined.

§ Various factors, such as nutritional state and availability of oxalacetate, of course, influence the extent of ketogenesis. Experimental conditions were, however, maintained rigidly constant in the experiments which are being compared.

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Fibrinogen Preservation in Serum After Heparin. (21399)

SAMUEL LOSNER AND BRUNO W. VOLK.

From the Department of Laboratories, Jewish Chronic Disease Hospital, Brooklyn, N. Y.

We reported recently that the interaction of calcium, fresh serum and homologous plasma rapidly produces a solid coagulum when carried out within one hour after blood withdrawal(1). If 50 mg of heparin was injected intravenously prior to blood removal, a firm clot was always obtained during interaction of fresh serum and homologous plasma. However, a dose of 75 mg of heparin, or greater, prevented formation of a solid coagulum. Since the amount of available fibrinogen in each plasma sample was practically identical, it appeared that the clot delaying action of heparin might be due to interference with the complete conversion of fibrinogen to fibrin as well as to its antithrombic properties. It was then assumed that fibrinogen could be preserved in serum after gross coagulation had taken place, despite presence of thrombin but provided a sufficient amount of heparin was administered previously. We, therefore, attempted to detect the presence of fibrinogen in serum obtained after gross coagulation of normal blood under the following conditions: a) in glass test tubes, b) in siliconated tubes, c) in tubes containing small amounts of heparin, d) in glass test tubes with blood from subjects who had received an intravenous injection of 50 or 75 mg of heparin. The conversion of blood from a free flowing to a gelatinous state was considered a valid criterion for gross coagulation(2). Clotting of a specimen of serum was considered sufficient evidence of the presence of fibrinogen. Coagulation after incubation of citrated serum with thromboplastin and addition of calcium, obviously represented a thrombinogenic process. Since fibrinogen is the plasma protein which forms fibrin under action of thrombin(3), its presence is proven if coagulation results from a thrombinogenic experiment.

In studies of the prothrombin consumption test, Quick(4) has shown that the thrombinogenic cycle is arrested by addition of sodium citrate to serum. In order to insure constancy of experimental procedure, sodium citrate was employed at given intervals as an agent stabilizing the prothrombin activity of serum.

Materials and methods. A. Twelve ml venous blood samples were withdrawn from 10 individuals and distributed in 3 ml aliquots in 4 labeled test tubes, and placed in water bath at 37°C. At the moment of coagulation, 0.3 ml of 0.1 molar sodium citrate was added to tube 1, and centrifuged immediately. This procedure was repeated at 10 minute intervals for tubes 2, 3 and 4. After centrifugation, prothrombin time of citrated serum sample was determined as indicated below. B. Same procedure with 12 ml blood samples from 6 normal individuals utilizing siliconated tubes, prepared according to Jaques(5). C. Similarly, 12 ml blood samples obtained from 10 normal individuals, were distributed in 3 ml aliquots into 4 test tubes each containing 12 γ of sodium heparin. After placing the tubes in water bath at 37°C, procedure was continued as under A. D. Twelve ml venous blood samples withdrawn from 10 individuals one hour after intravenous injection of 50 mg of heparin, distributed in 3 ml aliquots into 4 test tubes, and placed in water bath at 37°C. Procedure then followed as under A. E. Ten individuals received 75 mg of heparin each intravenously. One hour later 12 ml venous

blood samples withdrawn and procedure as under A. *Prothrombin time of citrated serum*: After centrifugation of the clotted blood to which sodium citrate had been added, 0.1 ml of citrated serum was placed in a microcuvette (75 x 6 mm) and prothrombin time determined after addition of Difco-thromboplastin and calcium according to photoelectric modification of the one-stage method(6). The difference in optical densities, before and after coagulation of citrated serum, as measured by magnitude of galvanometer lightbeam swing, was recorded as clot density. In previous studies(7,8) this value was a direct measure of fibrinogen concentration. In contrast to studies of prothrombin consumption, no deprothrombinized plasma or other fibrinogen containing material was added to serum in determining its prothrombin content, because serum contained sufficient fibrinogen to assure coagulation in most instances.

Results. A. In 6 out of 10 instances coagulation of normal citrated serum was observed in the first tube and then only within 15 to 30 seconds after the original clot had formed. All specimens taken at longer time intervals (10-30 minutes) remained fluid upon addition of thromboplastin and calcium to citrated serum thus indicating absence of detectable fibrinogen. Subsequent examinations of citrated serum from first tube at later intervals also indicated absence of fibrinogen. In 4 instances no measurable coagulation of citrated serum was obtained, indicating complete utilization of fibrinogen, almost at the moment of gross coagulation.

B. Similar experiments as in A but with blood obtained from 6 normal subjects and collected in siliconized material, produced clotting in the first tube in all instances, as well as in tube 2 to which citrate was added 10 minutes after gross coagulation. In one instance only did the third tube, to which citrate was added 20 minutes after clotting, reveal presence of fibrinogen. All prothrombin times were characterized by a marked lack of constancy, increasing in one instance from 17 to 46 seconds in tube 1 and from 22 to 60 seconds in tube 2, coincident with a declining clot density (Table I).

C. As seen in Table IIa *in vitro* addition of heparin (12 γ/3 ml of whole blood) led to preservation of fibrinogen for many hours after gross coagulation. Prothrombin times of citrated serum samples were characterized both by a considerable degree of reproducibility as well as constancy of time for all samples. However, fibrinogen concentrations, as indicated by clot density values, decreased steadily with time for the same sample. During observation period of three hours, all specimens obtained from the 4 tubes coagulated, thus indicating presence of fibrinogen.

D. *In vivo* administration of 50 mg of heparin to 10 individuals produced similar results as in C. Citrated serum from blood obtained one hour after heparin administration coagulated within 30 to 50 minutes after withdrawal. It revealed the presence of fibrinogen 3 hours after gross coagulation, as indicated by production of solid clot upon addition of thromboplastin and calcium. Presence of fibrinogen was demonstrated, in all but 2 instances, in the fourth tube to which sodium citrate was added 3 hours after gross coagulation (Table IIb).

E. After intravenous injection of 75 mg of heparin similar results were obtained as in D. All specimens of citrated serum revealed presence of fibrinogen without exception (Table IIc).

In separate experiments the supernatant citrated serum was decanted into another tube. A grossly visible coagulum became apparent subsequently.

The prothrombin times of citrated serum recorded in Tables I and IIa-c represent characteristic examples. Results in Tables IIa-c in different specimens varied from 10 to 13.5 seconds but remained relatively constant for each sample.

Discussion. It is generally assumed that almost any amount of thrombin however minute, can clot any amount of fibrinogen(9). However, it seems from our results that conversion of fibrinogen to fibrin may often be incomplete. This phenomenon can be observed for very short intervals when normal blood coagulates in glass test tubes, but as much as 20 minutes after gross coagulation occurs when siliconated tubes are used. Incomplete conversion of fibrinogen into fibrin

TABLE I. Prothrombin Times and Fibrinogen Concentrations of Citrated Serum Obtained from Normal Blood, Collected in Siliconated Material.

Time interval after gross coagulation, min.	Tube 1		Tube 2		Tube 3		Tube 4	
	0.3 ml of 0.1 M sodium citrate added to 3 ml of blood							
	Immediately after clotting		10 min. after clotting		20 min. after clotting		30 min. after clotting	
	P.T.*	d†	P.T.	d	P.T.	d	P.T.	d
0	17.0	2						
10	25.7	1.5	22.0	1				
20	35.1	1	42.0	1	fluid	—		
30	46.0	.5	60.0	.5	"	—	fluid	—

* Prothrombin time (in sec.) of citrated serum determined according to the One-stage-Quick method.

† Fibrinogen concentration expressed in terms of clot density wherein F (fibrinogen in mg %) = 18 d + 120.

TABLE II. Prothrombin Times and Fibrinogen Concentrations of Citrated Serum Obtained: (a) from normal blood to which 4 γ of heparin was added *in vitro*/ml.

Time interval after gross coagulation, hr	Tube 1		Tube 2		Tube 3		Tube 4	
	0.3 ml of 0.1 M sodium citrate added to 3 ml of blood							
	Immediately after clotting		1 hr after clotting		2 hr after clotting		3 hr after clotting	
	P.T.*	d†	P.T.	d	P.T.	d	P.T.	d
0	10.1	7						
1	11.9	4.5	12.0	5.5				
2	12.0	2	11.8	5	11.6	3		
3	12.0	1	12.2	3	11.8	2	11.8	4
	(b) from blood drawn 1 hr after intrav. inj. of 50 mg heparin							
0	13.7	8						
1	13.6	7	13.2	5.5				
2	12.7	6	13.3	4.5	12.9	4.5		
3	13.8	5.5	13.3	3.5	13.2	4	13.3	4.5
	(c) from blood drawn 1 hr after intrav. inj. of 75 mg heparin							
0	12.8	9						
1	13.0	6	12.9	6				
2	13.0	5	13.0	5	12.9	4		
3	13.2	4	13.0	4	12.9	3	13.0	4

* Prothrombin time (in sec.) of citrated serum determined according to the One-stage-Quick method.

† Fibrinogen concentration expressed in terms of clot density wherein F (fibrinogen in mg %) = 18 d + 120.

is readily demonstrable many hours after gross coagulation in the presence of heparin. Considerable amounts of fibrinogen are preserved in the supernatant citrated serum after coagulation and centrifugation. Since no gross coagulum was observed within the supernatant citrated serum, the conclusion appears warranted that the fibrin clot, at bottom of tube, acts as a core around which new fibrin molecules were polymerized during gradual disappearance of fibrinogen, manifested by a progressively declining clot density. This assumption was further borne out by formation of a solid coagulum after decanting of citrated serum.

It has been noted that viscosity of blood as measured with ultrasonic procedures increases steadily before and after the coagulation point (10). This may indicate that the process of conversion of blood fibrinogen into fibrin does not necessarily occur over a short time interval with maximum velocity, but extends over longer time periods. The physical phenomenon of gelation of blood then takes place during a relatively short phase as part of the more extended period of fibrinogen conversion.

The gradually declining clot density values (Table IIa-c), indicating a progressive decrease of fibrinogen concentration during the first 3 hours, must be taken as indication of some amount, however small, of active thrombin which should produce complete conversion of fibrinogen into fibrin(2). The fact that this conversion is incomplete, strongly suggests a direct inhibition by heparin. It has been shown that transition of fibrinogen into fibrin takes place as a two-step reaction(11), the first step constituting an activation of fibrinogen by thrombin, while the second represents actual polymerization. Since incomplete conversion of fibrinogen in the presence of heparin seems to be independent of available thrombin, the conclusion appears warranted that heparin may interfere with the second phase of the two-step reaction, namely the polymerization of fibrinogen.

Summary. 1. Very little or no fibrinogen remains after coagulation of normal blood in glass test tubes. 2. Small amounts of fibrinogen are detectable in siliconized tubes as late as 20 minutes after gross coagulation. 3. Considerable quantities of fibrinogen can be demonstrated in citrated serum from individuals having received 50 or 75 mg of heparin intravenously during a 3 hour observation period after gross coagulation has occurred. 4. Similarly, fibrinogen is detectable in citrated serum in considerable quantities after gross coagulation of normal blood when added to small amounts of heparin *in vitro*. 5. The preservation of fibrinogen in serum after heparin seems to be attributable to a direct inhibition of its polymerization reaction to fibrin.

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Plasma Derivatives in Tissue Cultures Intended for Growth of Poliomyelitis Viruses.* (21400)

P. L. BAZELEY, R. ROTUNDO, AND F. T. BUSCHECK.† (Introduced by Jonas E. Salk.)
From Virus Research Laboratory, School of Medicine, University of Pittsburgh.

In the multiplication of poliomyelitis viruses in tissue-culture, the nature and size of the cell population sensitive to virus infection is obviously of great importance. Factors which influence this, either in terms of the number of cells grown or the more efficient replication of virus from each cell, consequently are worth further study. Media consisting of serum and embryo extract in a solution of appropriate salts, can be devised to support good growth of active cells from monkey kidneys. However, for certain purposes, it may be desirable to obtain the final virus suspension in the simplest medium possible. This has, in part, been effected by the use of the synthetic Mixture 199 (Morgan, Morton, and Parker)(1) as employed by Wood *et al.*(2), and by Salk(3). Moderately active growth of cells may be obtained from monkey tissue

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† Acknowledgment is made for assistance in virus assay to Mr. R. Riley, and for general technical aid to Mr. L. Hall and Mrs. E. Jones.

in this medium, with useful virus yields following infection with each of the 3 poliomyelitis agents.

This communication presents information obtained from studies on addition of various plasma products to Mixture 199, both as regards the number of cells grown and the subsequent virus yields in cultures of monkey kidney tissue. The possibility of finding in plasma more specific growth substances for tissue culture than is present in whole serum was also considered in this approach.

Method. Tissue Culture. A. *Flasks.* Trypsinized cell suspensions were made by a method based on the work of Dulbecco and Vogt (4). From the final packed cell mass a 10% suspension was distributed in 2 or 5 liter diphtheria toxin flasks (Povitsky) containing 250 or 500 ml of Mixture 199, respectively. The cell doses varied in each trial and are given in the appropriate sections. Penicillin, 100 units/ml, and streptomycin, 100 units/ml, were added on all occasions. pH was adjusted by the addition of 2.8% $NaHCO_3$ solution (in distilled water) to give values between 7.6 and 7.8 when the stoppered flasks had been held at 36°C for 4 hours. The flasks were laid flat in the incubator for 3 days at 36°C; they could then be tipped daily to disturb the fluid without loosening cells adherent to the glass surface. In the flasks intended for virus growth, incubation proceeded for a total of 6 days without fluid change, by which time the initial pH of 7.6 to 7.7 had fallen to 7.1 to 7.2. Fluid was then drawn off and replaced by fresh fluid of the same composition. Incubation was carried on for a further 24 hours when the flasks were ready for virus seeding. Observations of growth were made visually under a dissecting microscope (at 48x magnification) and the extent of growth recorded as percent coverage of the surface available; changes of pH in the fluid were observed colorimetrically with phenol red. B. *Roller Tubes.* When roller tubes were used, one part of the 10% trypsinized cell suspension was added to 150 parts of Mixture 199, with pH readjusted and antibiotics as above. This was dispensed in 2 or 4 ml quantities in 15x150 mm Kimble screw-cap tubes, with rubber inserts cemented inside the caps. The 4 ml tubes were used for evaluation of the plasma derivatives and cell growth was followed by the same methods as used for the flasks. For assay of virus by cytopathogenic effect, the same type roller tube cultures, containing 2 ml of cell-medium mixture, plus 0.5% horse serum, heated at 56°C for 30 minutes, were placed at a slight angle in a stationary roller drum at 36°C for 6 days, by which time a confluent streak of epithelium had formed near the base. The fluid was now decanted and replaced by 4 ml of Mixture 199 only, adjusted to give a final pH of 7.6 to 7.8. Tubes were rotated a further 24 hours at 36°C before use. *Virus Cultivation.* When virus seeding was due, culture fluid was withdrawn from the flasks and replaced by a half volume of Mixture 199, *without* plasma additives, so that virus multiplication could not be affected by these factors. To the 250 ml in each 5 liter toxin flask, 0.5 ml of type I (Mahoney) virus fluid was added as infecting seed. The infective titer of this seed was $10^{-6.6}$/0.5 ml when assayed as described below. Harvests of virus fluids were made at 48 hours, the two flasks on each variable being pooled and a single sample assayed. *Virus Assay.* 0.5 ml quantities of virus dilution, made in Hanks' Solution(5), were added to each of 10 tubes at half log steps covering the active titration range; the tubes employed contained 4 ml of Mixture 199 and cell outgrowth, as described above. Readings of cytopathogenic effect were made microscopically at 6 days, the 50% end-point being determined by the probit method. The error of this assay is $\pm$ log 0.2, *i.e.*, preparations are significantly different when they vary two and one half times or more. Virus values are given in terms of relative potency compared with a type I standard preparation which, over more than 20 determinations, was found to have a mean 50% end-point of $10^{-6.3}$/0.5 ml. *Human Plasma Fractions.* These were kindly supplied by Dr. T. D. Gerlough of E. R. Squibb & Sons Co. with the kind permission of The American Red Cross. Each consisted of typical fractions prepared mainly by Method 6 (Cohn, *et al.*) (6) and were received by us in dried powder form. Solutions of either 2% or 5% were made in Hanks Solution and Seitz filtered be-

TABLE I. Cell Growth Observations and Virus Yields Obtained with Various Enhancing Factors.

Nutrient mixture	Dose of cell susp., ml	Changes of pH in growth fluid Day 4	Day 6	Epithelial growth (% coverage) Day 4	Day 6	Relative potency
M35 standard prep.						1.0†
S.M.* only	1.75	7.6	7.4	15	50	4.0
" "	3.50	7.5	7.3	20	80	8.0
S.M. + 0.5% horse serum	1.75	7.5	7.3	20	80	8.0
S.M. + 0.4% of 5% fraction IV (1349)	1.75	7.4	7.2	30	100	13.0
S.M. + 0.4% of 5% fraction IV (1358)	1.75	7.4	7.2	30	100	16.0
S.M. + 0.4% of 5% fraction IV (1358) + 0.5% horse serum	1.75	7.4	7.2	30	100	16.0

Note: pH of all preparations on Day 1 was 7.7.
* S.M. = Synthetic medium.
† 50% end-point of the standard as determined on this occasion was $10^{-6.4}$.

fore addition in appropriate amounts to the culture flasks or tubes. Two shipments of Fraction IV (1349 and 1358), in the original paste form as taken from the centrifuge, were received packed in dry ice. These were freeze-dried on arrival. Five percent solutions were then made, as above, from the resulting powder. These two batches, and also the one of Fraction V—(1321)—were fractionated from fresh human plasma collected without preservative. *Bovine Plasma Fractions.* Since human Fractions IV-1 and IV-3, 4, made from fresh plasma, were not available, their counterparts from bovine plasma were sought and kindly supplied in dried powder form by Dr. J. B. Lesh, of the Armour Research Laboratories. *Horse Serum.* The horse serum used was from active young horses, carefully bled. Weight expression of the clotted blood was performed in long test tubes, the product being removed the next day, after storage in the cold. This treatment produced a light straw-colored serum which was immediately centrifuged and Seitz filtered. Except where indicated, inactivation at 56°C for 30 minutes was carried out. *Human Serum.* A sample of freshly clotted human blood was centrifuged 3 hours after bleeding. The serum drawn off was immediately Seitz filtered and used without other treatment.

Experimental. Preliminary Search for Active Human Plasma Fractions. An attempt was first made to titrate a range of serum fractions for growth enhancement. Since these particular samples had been produced from reject plasma batches some years ago, it was not surprising that considerable toxicity for cultured cells was encountered in the screening test. Dilutions of each fraction in Hanks Solution, in a volume of 0.5 ml, were added to 10 roller tubes/variable. Each tube contained 4.0 ml of cell-medium mixture. Examination by microscope was carried out on the 4th, 6th, and 8th days. This procedure gave some indication that growth activity was related to the higher numbered fractions but it was not possible to test optimal concentrations because of increasing toxicity for the tissues. Further samples of more recent origin were obtained and tested in the same way. Fractions I, II, III, and III-2 from various sources appeared to have little activity.

Growth Promoting Activity in Human Plasma Fractions IV and V. Since growth enhancement appeared to be largely concerned with Fractions IV and V, these were then tested more thoroughly in 5 liter toxin flasks, containing 500 ml of medium, 2 ml of a 5% solution of each fraction being used. Comparisons were made also with the Synthetic Mixture 199 alone, and with horse serum added, 2.5 ml to a flask. Virus concentrations from each pool of two flasks (500 ml), for each variable, were also determined. The results are given in Table I. All 3 sets of preparations made with human plasma fractions showed rapid growth compared with the synthetic medium controls—and were equal to

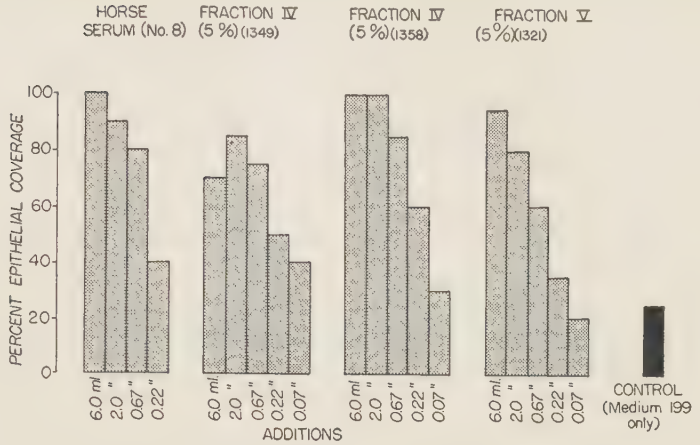

FIG. 1. Titration of growth enhancing value of horse and human plasma derivatives in monkey kidney tissue cultures (at 6 days).

or better than the flasks containing horse serum. In virus yield these same relationships held—there is a significant difference between the control preparation and those grown with plasma fractions. Both the horse serum and the double cell dose systems were found to occupy a position midway between these limits.

Titration of Growth Factors in Various Plasma Derivatives. The fractions and serum above were titrated and the results are given in Fig. 1. Human serum was included for control purposes. Similar preparations to those used above were employed except that 250 ml quantities of medium were placed in 2 liter flasks, with a cell dose of 1.0 ml of 10% suspension. The results confirm those found in Table I in respect to the amount of tissue grown with any particular additive. A second titration using the same method, but including *bovine* Fractions IV-1, and IV-3, 4, and V, as shown in Fig. 2, suggests strongly that the first and last of these 3 compounds stimulate growth (in this instance) while Fraction IV-3, 4 is almost inert.

Heat Stability of Active Plasma Components. Samples of plasma products were heated at 56°C for various periods of time, and tested by the same method in 2 liter flasks, again with 1.0 ml dose of 10% cell suspension. The results, seen in Fig. 3, show that Human Plasma Fraction IV loses the bulk of its activity rapidly, horse serum suffers an early partial loss, whilst Fraction V undergoes only slight alteration.

Discussion. Increased yields of virus have been obtained from a fixed amount of starting tissue, by the use of plasma fractions. Whether this is due to the greater number of cells avail-

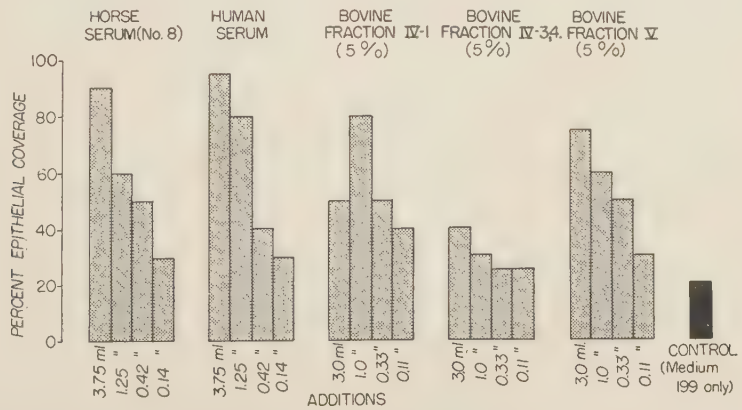

FIG. 2. Titration of growth enhancing value of horse, human and bovine plasma derivatives in monkey kidney tissue cultures (at 6 days).

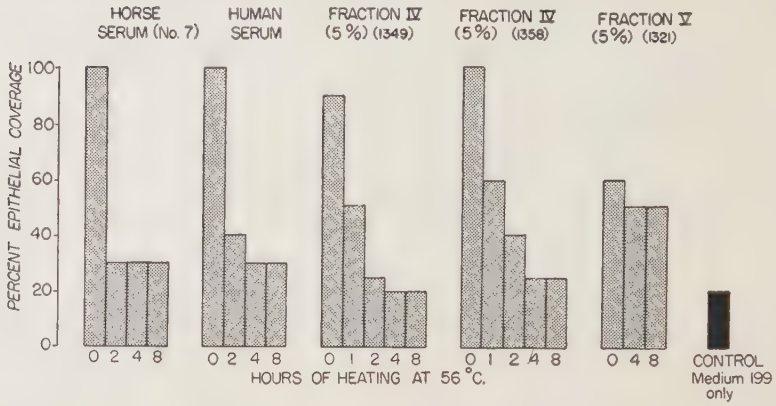

FIG. 3. Effect of heat (56°C) on cell growth enhancing substances in horse and human plasma derivatives (at 6 days).

able for infection, or to more efficient virus replication in cells multiplying very rapidly, has yet to be determined. Both factors may have played a part in the experiment described. From the data presented it is clear that one, or a mixture of defined plasma fractions, might be used in place of whole serum for growth enhancement of monkey kidney cells in the Synthetic Mixture 199. To secure optimal growth from a given number of cells originally, the added protein required from the fractions also is less than from whole serum, as might be expected. Economy in the use of parent tissue obviously can be secured by the addition of whole serum or plasma fractions to the synthetic Mixture 199. The advantage in the use of plasma fractions here is that additions may be of more specific substances than whole serum.

It is likely that 2 substances—a heat labile component of Fraction IV and the heat-stable Fraction V—are needed for optimal cell growth under these conditions. Further work in verifying the identity of the active components is necessary, if their use at optimal concentrations is to be achieved. It is of interest that neither Human Fraction IV or Fraction V appear to have any suppressive effect on virus multiplication when used in the manner described. Inhibition might well have been expected with some of the lower numbered human fractions due to their greater antibody content. Tissue growth, as described, appears to be concerned with the smaller molecular proteins of plasma occurring mainly at the end of the fractionation band. Some of these are by-products in present-day fractionation methods.

Summary. Plasma fractions added to synthetic Mixture 199 have been compared with whole serum for growth enhancement of monkey kidney cells in tissue culture. Fraction IV and/or V, when used in the concentrations given, appear to be capable of stimulating cell multiplication at least as well as whole serum. Yields of poliomyelitis virus were enhanced by the effect of these substances upon cellular growth.

Addendum. Since completion of this work, the validity of the surface cell coverage percentages in the flasks has been tested with total nuclei determinations of similar preparations, carried out by the method of Sanford, *et al.* (*J. Nat. Canc. Inst.*, 1951, v11, 773). Close agreement was found between percentage coverage of epithelium and the number of nuclei present in observations made during the periods of tissue culture recorded above.

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Some Quantitative Aspects of Passive Anaphylaxis in Pertussis-Vaccinated Mice. (21401)

Margaret Pittman and Frederick G. Germuth, Jr.*
From the National Institutes of Health, Public Health Service, Department of Health, Education, and Welfare, Bethesda, Md.

The mouse is relatively resistant to histamine and has low or irregular susceptibility to anaphylactic shock as compared with the guinea pig. Shortly after Parfentjev and Goodline(1) showed that pertussis vaccine was capable of inducing in the mouse a marked increase in susceptibility to histamine, Halpern and Roux(2) reported a slight but inconclusive increase in active anaphylactic shock in pertussis-vaccinated mice. Later, Malkiel and Hargis(3,4) and Malkiel, Hargis and Feinburg(5) showed that pertussis vaccine definitely increased active anaphylaxis in the mouse. Passive anaphylaxis was observed in a small number of pertussis-vaccinated mice by the latter authors(5) with the use of a 2 day interval between injection of antibody and antigen, while Kind(6) obtained a high rate of anaphylactic mortality when the antigen was given immediately following antibody transfer. Shortly before completion of the work to be reported in this paper, Muñoz *et al.*(7), in an abstract, reported a high percentage of fatal shock in pertussis-vaccinated mice challenged with bovine-serum albumin 4 to 6 hours after inoculation of a dose of 187 μg antibody nitrogen (rabbit serum); 48 hours after administration of the antibody the sensitivity for practical purposes had disappeared. Malkiel *et al.*(5) mentioned that Pittman had been successful in producing passive anaphylaxis.

The present paper is the first publication of the work to which they referred. It contains the results of determinations of the 50% antibody nitrogen (N) anaphylactic sensitizing dose (SD_{50}) of 2 sera in normal and pertussis-vaccinated mice and observations on the influence (a) of different amounts of pertussis vaccine and (b) of different time intervals between injection of antibody and antigen on the SD_{50}.

* Present address: Department of Pathology, Johns Hopkins Medical School, Baltimore, Md.

Materials and methods. Female mice(8) of the inbred strain NIH-BS, 4 to 5 weeks old and weighing 14 to 16 g at time of vaccination, majority weighing 16 to 18 g at time of anaphylactic testing, were used. The pertussis vaccine, formerly reference lot no. 4, is now the United States Standard. For NIH-BS female mice, 5 days after injection of 2.5×10^9 bacteria, the LD_{50} of histamine diphosphate is around 30 mg/kg (LD_{50} for normal mice is 1750 mg/kg or higher). Protective activity of 8×10^9 bacteria equals 1 U. S. unit(9). Per bacterium, this activity is the lowest acceptable for release of lots by the National Institutes of Health. The two lots of anti-bovine-serum-albumin rabbit serum contained 0.816 and 2.52 mg of antibody N per ml, respectively. The latter serum was furnished by Mr. J. Oyama of this Institute. The passive anaphylactic SD_{50} of a serum was determined as follows: mice were given intraperitoneally 2.5×10^9 bacteria of the vaccine and 3 days later inoculated intravenously with varying amounts of antibody N in a volume of 0.1 ml per 10 g weight, unless a larger volume was required to supply the desired amount. Then 48 hours after the antibody and 5 days after vaccination each mouse was given intravenously 1 mg of crystalline bovine-serum albumin in 0.5 ml of warm saline. Non-vaccinated mice were inoculated similarly with the antibody and antigen. Additional doses of 0.625×10^9 and 10×10^9 bacteria were used to determine the influence of different amounts of vaccine on anaphylactic shock. In the final experiment, time intervals of 48, 24 and 6 hours between antibody and antigen were used; the 5 day interval between pertussis vaccination and antigen challenge was kept constant. Histamine susceptibility of control vaccinated mice was determined on the day of anaphylactic test. Varying doses of histamine diphosphate (38.18% histamine base), dissolved in dis-

TABLE I. Lot No. 1 Anti-Bovine-Serum-Albumin Serum: Titration of Passive Anaphylactic Sensitizing Dose and Influence of Amount of Pertussis Vaccine.

Exp.	Antibody,* mg N/kg	Pertussis vaccine—No. of bacteria: .625 × 10^9	2.5 × 10^9	10 × 10^9	No vaccine
		Anaphylaxis—1 mg bovine-serum albumin			
1	16		17/18†		7/15
	4		17/27		0/12
	1		2/26		
	0.25		0/21		
	SD_{50} mg N/kg		3.3 (2.3, 4.6)		>16
2	24				5/16
	12				2/16
	6		12/16		0/16
	3		4/16		
	1.5		0/16		
	SD_{50} mg N/kg		4.3 (3.4, 5.4)		32.4 (16.2, 59.2)
3	32				3/13
	16	4/14			5/16
	8	5/6	14/15		0/16
	4	4/16	4/16	7/16	
	2		3/16	1/16	
	1			0/16	
	SD_{50} mg N/kg	?	4.4 (3.4, 5.7)	4.3 (3.2, 5.2)	>32
		Histamine diphosphate LD_{50}, mg/kg			
1		50 (30, 84)			
2		Not determined			
3		133 (68, 266)	33.5 (26, 44)	18.2 (14, 24)	

Experiments were performed in fall of 1951. Limits of 2 S. D. for 50% doses are given in parentheses.

* Antibody nitrogen content of serum = .816 mg/ml.

† No. of shock deaths/No. of mice injected.

tilled water, were injected intraperitoneally in 0.1 ml per 10 g weight of mouse. The onset of anaphylactic or histaminic shock occurred within a few minutes and death usually followed within 30 minutes. The majority of mice that showed severe shock, died. Only deaths, including the few that died overnight, are reported in the results.

Results. It is shown in Tables I and II that pertussis vaccine induced a significant increase in susceptibility of the mice to passive anaphylaxis; that the response was graded in relation to amount of antibody N administered, that reproducible titrations of the SD_{50} of each serum were obtained; and that within the 16-fold range of vaccine dosage, the SD_{50} was not significantly affected. The mean of the 4 SD_{50} values of no. 1 serum, obtained with mice vaccinated with 2.5 x 10^9 and 10 x 10^9 bacteria, is 4.06 ± .27 mg N/kg while the mean of the 5 values of no. 2 serum, obtained with mice similarly vaccinated, is 7.9 ± 1.03 mg N/kg. A sufficient amount of no. 1 serum was not available to give to all mice vaccinated with 0.625 x 10^9 bacteria. With no. 2 serum in no. 3 experiment, there was a suggestion of a slight decrease in size of the SD_{50} as the dose of vaccine increased but the difference is not significant.

In contrast, with non-vaccinated mice, a low incidence of poorly graded anaphylactic shock was obtained. In the 3 experiments with no. 1 serum, the fatalities of mice that received 16, 24 and 32 mg N/kg were 47, 31 and 23%, respectively. With no. 2 serum and doses of 16, 24 and 24 mg N/kg, the fatalities were 50, 48 and 55%, respectively.

Also shown in Tables I and II, are the 50% lethal doses of histamine for control vaccinated mice in 5 of the 6 experiments. Examples of titration are given in Table II. In each table it is shown that variation in amount of vaccine had a greater influence on susceptibility to histamine than to anaphylaxis. Comparable histamine LD_{50} values were obtained in the respective tests in the two Tables. These were performed about 2½ years apart. The average, 31.2 mg/kg, of the 3 LD_{50} values obtained

TABLE II. Lot No. 2 Anti-Bovine-Serum-Albumin Serum: Titration of Passive Anaphylactic Sensitizing Dose and Influence of Amount of Pertussis Vaccine.

Antibody,* mg N/kg	Pertussis vaccine—No. of bacteria: .625 × 10^9		2.5 × 10^9		10 × 10^9		Saline, .5 ml	
Anaphylaxis—1 mg bovine-serum albumin								
			No. 1				No. 1	
16			15/20†				10/20	
8			16/20					
4			8/20					
2			0/20					
SD_{50} mg N/kg			4.5 (2.4, 8.5)					
	No. 2	No. 3	No. 2	No. 3	No. 2	No. 3	No. 2	No. 3
24							7/16	11/20
12	10/16	15/20	14/16	13/20	9/16	16/20	6/16	8/20
6	4/16	3/20	3/16	7/20	3/16	6/20	1/16	2/20
3	0/16	0/20	0/16	3/20	1/16	2/20		
SD_{50} mg N/kg	9.3	9.2	8.1	8.4	10.9	7.6	24.5	19.1
Combined	9.3 (7.9, 10.9)		8.2 (6.9, 9.6)		8.9 (7.2, 11)		21.5 (14.5, 32)	
Histamine diphosphate susceptibility‡								
(mg/kg)								
75	8/16†		12/16	14/20	14/16			
37.5	1/16		13/16	12/20	13/16			
18.75	1/16		6/16	7/20	6/16			
LD_{50}, mg/kg	79.4 (52, 120)		28.7 (18, 47)	30.5 (18, 52)	22.4 (15, 34)			

Experiments were performed in Mar. and Apr., 1954. Limits of 2 S. D. for 50% doses are given in parentheses.

* Antibody nitrogen content of serum = 2.52 mg/ml.

† No. of shock deaths/No. of mice injected.

‡ LD_{50} for control vaccinated mice in No. 1 = 34.4 (19, 44) mg/kg.

in 1954 with mice treated with 2.5 x 10^9 bacteria, is the same as the average, 30.6 mg/kg, of the LD_{50} values of 7 tests performed in 1951.

In Table III it is shown that the SD_{50} values obtained when serum no. 2 was given either 48, 24 or 6 hours before the antigen, were not significantly different.

Discussion. The results presented show that pertussis vaccine significantly increased susceptibility of mice to passive anaphylaxis. Reproducible titrations of the 50% sensitizing dose of each of two sera, containing 0.816 and 2.52 mg antibody N/ml, were obtained but it appears that the SD_{50} of the respective sera may be different. The mean SD_{50} values, which are significantly different at the 5% level, were 4.06 ± .27 and 7.9 ± 1.03 mg N/kg, respectively. The mean values, however, were derived from values obtained with mice vaccinated with either 2.5 x 10^9 or 10 x 10^9 bacteria. No difference in anaphylactic response of the mice in the two groups was detectable. In addition, titrations of the respective sera were performed 2½ years apart. During this time, there may have been a decrease in the anaphylactic stimulating factor of the vaccine. There was, however, no detectable diminution in the histamine sensitizing property of the vaccine. Further experimentation will be required to determine if sera of low or high antibody content vary in passive sensitizing property. A shortage of no. 1 serum prevented a direct comparison with no. 2.

The amount of antibody N required to induce a high percentage of fatal shock may be similar to the amount reported by Muñoz *et al.*(7). The dose they employed, 187 μg per mouse, would be equivalent to 9.3 mg/kg for a 20 g mouse. They reported, however, that a time interval greater than 6 hours was impractical for sensitization, whereas we obtained comparable SD_{50} values when the antibody was given from 6 hours to 48 hours before the antigen. Lack of published details of their work prevents further comparison of the respective studies.

Within the range of dosage of the pertussis vaccine and under the other conditions em-

TABLE III. Influence of Different Time Intervals between Injection of Antiserum and Albumin.

Antibody, mg N/kg	Serum inj. before albumin 48 hr	24 hr	6 hr
12	13/20*	16/20	16/20
6	7/20	11/20	8/20
3	3/20	2/20	2/20
SD_{50} mg N/kg	8.4	6.3	7.1
2 S. D. limits	5.7, 12.4	4.9, 8.1	5.5, 9.1

Test formed a part of No. 3 reported in Table II.

Time interval between pertussis vaccine and albumin injections was 5 days.

* No. of anaphylactic deaths/No. of mice injected with 1 mg bovine-serum albumin.

ployed, the amount of vaccine required to induce the maximum histamine susceptibility was not required to induce the maximum passive anaphylactic shock. The SD_{50} values of no. 2 serum, experiment no. 2, for mice vaccinated with the 3 doses of vaccine, 0.625 x 10^9, 2.5 x 10^9, and 10 x 10^9 bacteria, were 9.3, 8.1 and 10.9 mg N/kg respectively while the LD_{50} values of histamine diphosphate were 79.4, 28.7 and 22.4 mg/kg respectively. With either phenomenon, it appears that additional amounts of vaccine above the minimum effective dose, have little effect on increasing susceptibility to shock. It might be anticipated, however, that the anaphylactic reaction like the histamine reaction would be affected by the strain of mice(10) and the activity of the lot of vaccine. Vaccines of high protective potency tend to be high in histamine sensitizing activity(11).

The findings that treatment with pertussis vaccine increases the susceptibility of the mouse to both histamine and anaphylactic shock is of considerable interest in regard to the histamine theory of the mechanism of anaphylactic shock. However, until there is a better understanding of the cause of the pertussis-vaccine induced histamine sensitivity in the mouse, it might be better to reserve interpretation of the present findings in support of the role of histamine in anaphylaxis. The phenomenon of increased susceptibility in the mouse does not occur in pertussis-vaccinated rabbits and guinea pigs. On the contrary, the vaccine induces a slight decrease in histamine sensitivity(12).

It is indicated that the histamine sensitizing and anaphylactic stimulating properties of *Haemophilus pertussis* for mice, which have not been found in other bacteria except *Brucella abortus* and then to a lesser degree(4), may make possible the use of mice in anaphylactic studies.

Summary. 1. Pertussis vaccine significantly increased the susceptibility of mice to passive anaphylaxis. Two lots of anti-bovine-serum-albumin rabbit serum containing 0.816 and 2.52 mg antibody N per ml, respectively, were used. With each, the response was graded in relation to amount of antibody injected and reproducible titrations of the SD_{50} were obtained. The SD_{50} values for the respective sera were 4.06 $\pm$ .27 and 7.9 $\pm$ 1.03 mg N/kg, respectively. Significance of the difference was not definitely established. In non-vaccinated mice, even with several fold increases in antibody, the highest incidence of shock was around 50% and frequently the response was irregularly graded. 2. Within a range of dosage of pertussis vaccine that varied as much as 16 fold and which significantly affected the histamine LD_{50}, the passive anaphylactic SD_{50} values of the sera were not significantly altered. 3. Comparable anaphylactic sensitivity was observed when a serum was administered either 48, 24, or 6 hours before the antigen. Besides the theoretical significance, the findings indicate that mice treated with pertussis vaccine may be useful in anaphylaxis studies.

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Human Type Caries Distribution in Osborne-Mendel Rats Kept on Heated Skim Milk Powder Diet.* (21402)

F. L. Losee and J. L. Nemes. (Introduced by F. P. Ellinger.)

From the Naval Medical Research Institute, National Naval Medical Center, Bethesda, and Department of Bacteriology, University of Maryland.

This laboratory has endeavored, by dietary means, to produce a surface and occlusal caries pattern similar to the human in the Osborne-Mendel rat. Schlack, *et al.*(1) reported high susceptibility of this strain (NMRI† colony) of white rats to pit and fissure (occlusal) caries only. Surface and occlusal lesions in Sprague-Dawley rats fed a skim milk powder diet have been reported by Stephan and co-authors(2,3,4). Typical surface and some atypical occlusal caries lesions have been developed in the Holtzman rat by McClure(5). Later, McClure and Folk(6) reported surface lesions in 84% and occlusal caries lesions in 5% of Sprague-Dawley and Holtzman rat strains. The diet contained heat processed commercial skim milk powder and permitted slight weight gain in the animals.

The present study was undertaken to ascertain whether this diet would produce occlusal *and* surface lesions in the Osborne-Mendel strain.

Experimental. Osborne-Mendel rats were maintained on unsupplemented Rockland rat diet for the complete life cycle of the breeder strains‡, including suckling period of the experimental animals which, when weaned, were placed on the test diet at 20-22 days. The McClure-Folk diet No. 636 consisting of skim milk powder§, 35%; corn starch, 45%; cerelose, 18%; liver powder, 2% and a vit. A, D and E concentrate were prepared in the laboratory at the NIDR to reduce experimental variables. The diet and distilled water (NMRI source) were available *ad lib.*; vitamin concentrate was fed to animals by mouth one drop per week. Animals were kept in stainless-steel, screen bottomed cages, two animals of same sex per cage and the cages not cleaned for duration of the test. Animals were housed in a tiled room at 74-77° F, relative humidity of 50-69% with one strain of rodents in the room. A litter of 12 animals from an occlusal caries-resistant female was used, 4 animals to a cage, to assess surface caries susceptibility of offspring. Maintained for 61 days on this diet, the animals were sacrificed, heads removed, autoclaved, cleaned of adhering soft tissue and the jaws kept in water at 5°C. Charting, incidence and severity of caries were evaluated by the method of McClure and Folk at NIDR and confirmed at NMRI. All occlusal surfaces were ground by the method of Schlack, *et al.*(7) to expose the carious lesions otherwise hidden within the coronal grooves. Our observations on the Osborne-Mendel rat strain are presented in Table I together with pertinent data from the McClure and Folk study on Sprague-Dawley and Holtzman rat strains.

Results. Table I shows a 97% caries susceptibility in the NMRI rat colony reflecting a slight increase in number of rats with surface caries but a 17 fold increase in susceptibility to occlusal lesions over the reference study. This high susceptibility to occlusal *and* surface lesions among the Osborne-Mendel rats approximate human caries distribution more closely than any data known to the authors.

In the NMRI rats, the same number of teeth had occlusal as surface caries, but the incidence of occlusal carious areas was 20 times as large as in the Sprague-Dawley and Holtzman strains of the NIDR.

The score per carious rat was a reflection of

* The opinions or assertions contained herein are the private ones of the writers and are not to be construed as official or reflecting the views of the Navy Department or the naval service at large.

† Naval Medical Research Institute.

‡ National Institute of Dental Research (NIDR) stock colonies use Purina lab chow, greens twice weekly supplemented with bread and fluid milk, available to mother and offspring during lactation.

§ Roller process, autoclaved 15′-121°C in half inch layer between pans sealed with adhesive tape with no exposure to moisture.

TABLE I. Comparison of Results from Feeding Diet No. 636 on Caries Experience of Young Albino Rats Using NIDR Scores.

Method of examination	Sprague-Dawley and Holtzman strains*	Osborne-Mendel strain		
	Clinical	Clinical	Clinical + grinding	Clinical + grinding
No. of rats	38	36†	36	12‡
" " litters	29	18	18	1
Days on experiment	90	61	61	61
Initial wt	26.2	28.7	28.7	24.8
Final "	46.7	153.9	153.9	128.6
Avg daily gain	0.23	2.06	2.06	1.70
	Caries incidence, %			
Carious rats	84.2	97.2	97.2	100.0
Occlusal carious rats	5.3	69.5	86.1	0.0
Surface " "	84.2	91.6	91.6	100.0
	Caries distribution and severity—No./rat			
Upper carious teeth	.13	.08	.30	.00
Lower " "	2.82	4.11	4.33	2.83
Total " "	2.95	4.19	4.63	2.83
Occlusal " "	.11	2.47	3.02	.00
Surface " "	2.89	2.58	2.58	2.83
Occlusal " areas	.13	2.52	3.22	.00
Lingual " "	.05	.00	.00	.00
Buccal " "	4.08	3.97	3.97	4.66
Score/rat	7.11	8.72	9.38	5.50
Score/carious rat	8.44	8.97	9.65	5.50

* McClure and Folk op. cit. † Equal No. of males and females. ‡ Litter from an occlusal caries resistant female.

lesion incidence as well as severity. Although scores were similar in the NMRI and NIDR studies, it was felt that the scoring method minimized the large number of occlusal carious areas in the NMRI studies. The score for the NMRI series might have been greater if the experiment had continued to the 90 day period of the NIDR studies.

Evaluation of occlusal carious areas by the grinding method resulted in a 20% increase over clinical examination procedures and suggested this refinement of technic as essential for precise enumeration of early occlusal type one caries characterized by enamel invasion without dentoenamel junction involvement.

Average weight gain of rat colonies when supported on nutritionally deficient diet No. 636 should be noted. Although the incidence of surface caries was similar in both studies, the NIDR animals had an average weight gain of 0.2 g per day whereas the NMRI animals maintained a gain of 2.0 g per day. The relationship between body weight and caries will be discussed in a later publication.

Finally, attention may be called to the litter from an occlusal caries-resistant female from the NMRI colony. Although devoid of occlusal caries, as expected, this litter developed surface caries of the pattern and severity comparable to the caries susceptible NMRI colony. This result suggests strongly that the surface type of caries as developed by diet No. 636 is separate and independent from the occlusal type caries.

Summary. Dental caries with occlusal and surface lesions approximating human caries distribution has been produced in the Osborne-Mendel strain of the white rat on heated skim milk powder diet of McClure and Folk. The lesions were developed fully in 97% of the animals at the age of 81 days. The data presented suggested independent etiologies for the surface and occlusal types of caries lesions.

The technical assistance of David E. Westerman of NMRI and John D. Rust of NIDR is acknowledged with thanks.

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Differential Inhibition of Virus Hemagglutination by Chlorophyllin. (21403)

WOLCOTT B. DUNHAM.

From the General Medical Research Laboratory, Veterans Administration Medical Teaching Group Hospital, Memphis, Tenn.

Numerous compounds have been found to inhibit hemagglutination (HA) by viruses (1,2,3,4). That chlorophyllin inhibits HA by certain viruses, to markedly different degrees, is shown by results of the present studies. This agent was found to inhibit in increasing order the HA produced by influenza, mumps and Newcastle disease viruses (IV, MV, NDV).

Materials and procedures. Viruses used were NDV kindly furnished by Dr. F. R. Beaudette, strain EMW of MV kindly supplied by Dr. John Enders and IV, strain PR8. In most experiments infected allantoic fluid (AF) was used as the virus suspension. In some tests partially purified suspensions were employed, obtained by precipitation of virus from AF with methanol followed by elution of virus from washed precipitate(5). Serial, 2-fold virus dilutions were used throughout. Except as indicated, all solutions and suspensions were prepared in M/200 phosphate buffered physiological NaCl solution (BSS), pH 7.23. Adult chicken blood was drawn into BSS. The red blood cells (RBC) were washed twice and 1% suspensions prepared. Solutions of 11 copper chlorophyllin preparations were employed. At low concentrations, all of these affected virus HA similarly. However, at final concentrations of 0.25 mg/ml all but lot 54 produced in controls varying degrees of hemolysis or agglutination. Lot 54 was used in all experiments described in this report. HA inhibition tests consisted of 0.25 ml chlorophyllin solutions, 0.25 ml 1% RBC suspension and 0.5 ml virus dilutions introduced in various orders into 12 x 75 mm tubes. Tests were read by observing the film (++), ring (+) or button (O) at the bottom of tubes after remaining at room temperature 45 or 60 minutes, the period being uniform in replicate experiments. The highest dilutions of virus producing films were considered to contain 1 HA unit in the volume used. Inhibition was determined by comparison with simultaneous control titrations of virus with BSS in place of chlorophyllin. Values reported are averages of those obtained in at least 6 tests.

Results. Effect of Chlorophyllin on Virus HA. Block tests were performed with all combinations of 4 concentrations of chlorophyllin and 9 dilutions of virus. RBC were mixed with chlorophyllin, and virus added 15 to 25 minutes later, the time required for pipetting. HA by the 3 viruses was inhibited to different extents, 0.125 mg chlorophyllin inhibiting an average of approximately 0.5 HA unit of IV, 1.5 units of MV and more than 16 units of NDV (solid lines, Fig. 1). The differences of the means of observed inhibitory activity of 0.25 mg chlorophyllin on the 3 viruses are highly significant, $P<0.0001$ for each of the 3 possible combinations of viruses.

Action of Dialyzed Chlorophyllin on Virus HA. Aqueous solutions of chlorophyllin were dialyzed at 4°C for 24 hours against 3 changes of water totaling 26 volumes. The pooled dialysate was concentrated by evaporation at 80°C, made up with water and concentrated BSS so that each ml represented 40 times the amount of chlorophyllin in the most dilute

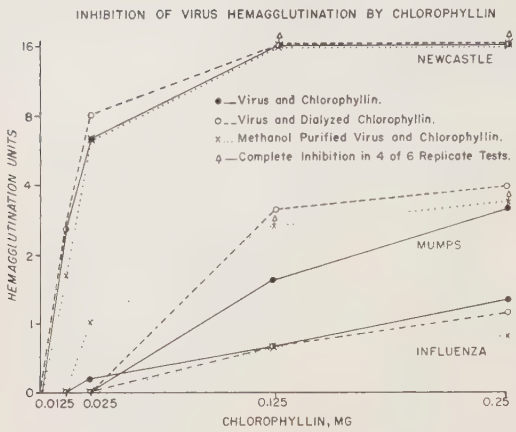

FIG. 1.

solutions used, a concentration that inhibited an average of 2.7 HA units of NDV. The dialysates had no effect on HA by any of the viruses. The dialyzed chlorophyllin inhibited virus HA to a degree similar to that produced by undialyzed aliquots (broken lines, Fig. 1).

Action of Chlorophyllin on HA by Purified Viruses. Chlorophyllin inhibited the HA produced by viruses purified by methanol precipitation. The extent closely approximated that obtained with unpurified, allantoic fluid suspensions of the 3 viruses (dotted lines, Fig. 1). In some experiments with purified MV, which had a low HA titer, and with NDV, which was strongly influenced by chlorophyllin, the end point of inhibition was not demonstrated as there was inhibition at all virus dilutions tested (△ Fig. 1). The lowest dilutions in each of these instances, however, may have represented the actual end points as replicate tests with more concentrated virus gave definite values which were comparable.

Influence of Period of Exposure of Virus to Chlorophyllin. Chlorophyllin was mixed with aliquots of serial virus dilutions and allowed to stand for ½ minute to 60 minutes before RBC were added. Control titrations that had stood for the same periods with BSS in place of chlorophyllin were used for reference. Because of the differences in the reactivity of viruses with chlorophyllin, it was necessary to use a different concentration of chlorophyllin with each virus, the maximum with IV but only 1/25 as much with NDV, to avoid complete inhibition of HA. The inhibition increased with the period of exposure of virus to chlorophyllin (Fig. 2).

pH Controls. Each virus was titrated simultaneously in the presence of BSS and M/20 Na_2HPO_4, pH 8.9. The pH of test mixtures in these parallel series was 7.23 to 7.40 and 7.81 to 7.94. The end point of agglutination was identical in both series. The pH of all test materials fell within this range, indicating that the pH was not a factor in any of the results. Also, virus dilutions exposed for 60 minutes to this alkaline buffer and to BSS gave identical HA patterns.

Discussion. Chlorophyllin is at least 16 times more active in inhibiting HA by NDV than by IV. This is in contrast to the action of purified urinary mucoprotein which is much more effective against IV than against NDV (6).

Certain other reactions are influenced by chlorophyllin, but no reports have appeared on its action on viruses. Barnard, Goldman, Kessler and Stanton(7) have shown that chlorophyllin inhibits agglutination of human RBC by type specific antisera. They have

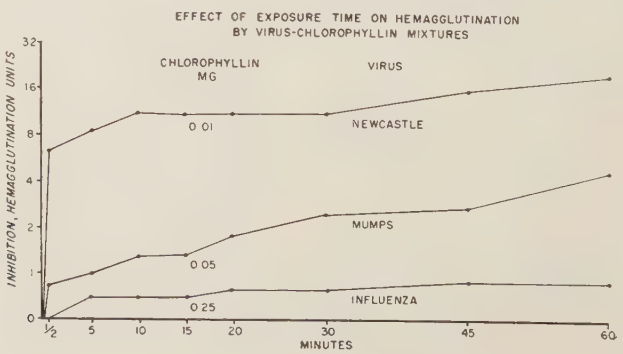

FIG. 2.

also presented evidence that chlorophyllin delays clotting of blood and that it inhibits the action of hyaluronidase.

At least 2 processes may be involved in chlorophyllin inhibition of HA by NDV and MV. One takes place so rapidly that its rate and site of action can not be detected by the technique used. It is manifest in the tests in which dilutions of NDV and MV were mixed with chlorophyllin and the RBC added ½ minute later (Fig. 2). The other is a much slower reaction. At 10 minutes inhibition was only about twice that effected in ½ minute. It appears to be a direct action on the virus itself, the extent of inhibition being dependent on the period of time that the virus and chlorophyllin are in contact before the RBC are added. This indicates that chlorophyllin may also partially inactivate these viruses since, as pointed out by Hirst(8), agents that decrease the HA properties of viruses also have been found to decrease their infectivity.

Summary. Copper chlorophyllin inhibited hemagglutination by influenza A, mumps and Newcastle disease viruses. This action was not due to a dialyzable component. Hemagglutination by Newcastle disease virus was most affected. Part, at least, of the inhibition appeared to be due to a direct effect on the virus.

The author wishes to express appreciation to Mr. Henry T. Stanton, Jr., of Rystan Co. for furnishing samples of chlorophyllin, to Mr. Stanton and Dr. Robert D. Barnard for valuable information and to Mr. Samuel Goodloe for excellent technical assistance.

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Oxygen Uptake of Human Erythrocytes in Fresh and Stored Blood.* (21404)

RAYMOND F. SHEETS, HENRY E. HAMILTON, AND ELMER L. DEGOWIN.
(With the technical assistance of Richard H. King.)
From the Department of Internal Medicine, College of Medicine, State University of Iowa, Iowa City.

Harrop(1) was able to measure the oxygen consumption of reticulocytes, but he was unable to demonstrate a similar phenomenon in mature normal human erythrocytes with the apparatus available at the time. More recent workers have measured oxygen uptake in nonnucleated cells. Wright(3) showed that the oxygen uptake of avian erythrocytes was one to 4 times that of human red blood cells. Michaelis and Solomon(2) demonstrated that the oxygen consumption of mammalian erythrocytes was increased by the addition of methylene blue. Subsequently it was shown that plasma took up oxygen and that a portion of the oxygen uptake of whole blood could be attributed to the plasma lipids and proteins (4,6,7). Various aspects of the oxygen consumption of erythrocytes from several animals have been studied(5,8-12). Bird(14) has noted that the addition of glucose has no effect on the oxygen consumption of human erythrocytes. The present studies were made in the course of a search for clues to the better preservation of human blood for transfusion.

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Methods. Manometric method. All sam-

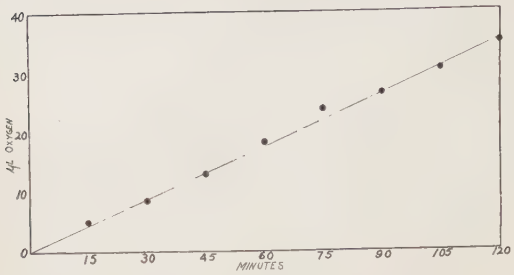

FIG. 1. Typical curve obtained in measurement of oxygen uptake of human erythrocytes. Each solid circle represents avg uptake of 6 manometers at intervals of 15 min.

ples of blood were rotated gently in Erlenmeyer flasks for 10 minutes to permit complete oxygenation of the hemoglobin and plasma. Three ml of blood mixture were then placed in a Warburg vessel and 0.2 ml of 20% KOH was placed in the center well. The vessel was attached to a Warburg manometer in the usual manner and immersed in a water bath maintained at 37 ± 0.5°C. The manometers were shaken at the rate of 120 strokes per minute. The stopcocks were left open for 15 minutes to permit equilibration, then they were closed and the period of observation began. The manometers were read every 15 minutes for 2 hours. Exploratory runs were continued for 5 hours but showed no significant alteration in the rate of oxygen uptake in the last 3 hours. The data from a typical single run in a manometer are plotted in Fig. 1. The erythrocytes were counted by the procedure previously described and discussed(13). In these studies the oxygen consumption is expressed as microliters of oxygen per 10^9 erythrocytes per hour. *Fresh blood.* Fresh blood was drawn from the donor with a clean, dry syringe and needle and placed in an Erlenmeyer flask with one drop of 25% sodium citrate solution per 5 ml of whole blood. In certain experiments one mg of heparin (Connaught Laboratories, Toronto) per 5 ml of blood was substituted for the sodium citrate. *Stored blood.* Five hundred ml of blood were drawn from each of 9 donors into Baxter vacuum bottles. The bottles contained 120 ml ACD solution. Each 100 ml of ACD solution contained 1.53 g dextrose, U.S.P., 1.38 g sodium citrate, U.S.P., and 0.50 g citric acid, U.S.P. The bottle was gently agitated until mixing was complete. An aliquot of blood mixture was then removed with aseptic technic and the oxygen uptake was measured without delay. In the interval between determinations the blood was stored without agitation in a refrigerator at 4°C. *Accuracy of method.* The value for the oxygen uptake was the average from 6 manometers containing the same blood mixture. The standard deviation of the oxygen uptake in microliters per 10^9 erythrocytes was computed from the measurements on 51 samples of blood from males to which either sodium citrate or heparin was added, using the equation:

$$\sigma = \frac{\sqrt{\Sigma\ (x - \bar{x})^2}}{N}$$

where N = the number of samples, x = the oxygen uptake of each sample in microliters per 10^9 erythrocytes per hour (average of readings of 6 manometers), $\bar{x}$ = mean of the values for oxygen uptake, σ = standard deviation. The coefficient of variation of the oxygen consumption of the 51 samples was 19%, but this wide variability includes differences between individual bloods as well as errors in measurement. Further calculations were made to evaluate the accuracy of the technic itself. The standard deviation of the volumes of oxygen consumed among the 6 manometers used in a single study were calculated from

$$\sigma y = \frac{\sqrt{\Sigma\ (y - \bar{y})^2}}{M}$$

where y is the value for a single measurement in one study, and M = 6. The standard error of the determination is the S. E. of the mean of the 6 measurements:

$$\text{S. E.} = \frac{\sigma y}{\sqrt{M - 1}}$$

To obtain the "average" accuracy the mean square standard error was computed:

$$(\text{MSSE}) = \frac{\sqrt{\Sigma\ (\text{SE})^2}}{N}$$

where N is now 51. The mean square standard error is .019 or 1.8%. It takes 2.57 standard errors to embrace 95% of the cases in samples as small as 6. Hence the error of the method is ± 4.6%. This method of

TABLE I. Oxygen Uptake of Fresh Human Blood.

No. of subjects—sex		Avg μl $O_2/10^9$ cells/hr	Range μl $O_2/10^9$ cells/hr	Range red cell count, $10^6/mm^3$	Anticoagulant
28	♂	1.0412	.7564-1.5583	4.62-6.74	Sodium citrate
23	♂	1.0497	.5124-1.3464	4.56-6.00	Heparin
23	♀	1.2704	.7481-1.5369	3.92-5.49	Sodium citrate

analysis eliminates the differences encountered due to variations in individual blood samples and indicates more accurately variations to be expected due to inaccuracies in the technic.

Results. Men's blood with sodium citrate. (Table I) Blood was collected from 28 normal male medical students, residents, and interns and each sample was measured in 6 manometers. The average oxygen uptake for all samples was 1.0412 μl per 10^9 erythrocytes per hour, with the range from 0.7564 to 1.5583 μl. The red cell count of the samples varied from 4.62 millions to 6.74 millions per cu mm.

Men's blood with heparin. (Table I) The heparinized blood of 23 donors was studied with 6 manometers for each sample. The average oxygen uptake per 10^9 erythrocytes per hour was 1.0497 with the range from 0.5124 to 1.3464 μl. The red cell count varied between 4.56 millions and 6.00 millions per cu mm. The same donors also furnished blood for the previous study and the average oxygen uptake in each study was 1.04 μl per 10^9 cells per hour. From this we concluded that sodium citrate as an anticoagulant was as satisfactory as heparin for the determination of oxygen uptake.

Women's blood with sodium citrate. (Table I). In this group 23 young normal women served as donors. Six manometers were used in the study of each blood and the range was from 0.7481 to 1.5369 μl of oxygen uptake per 10^9 cells per hour with an average for this group of 1.2704 μl. The red cell count varied between 3.92 millions and 5.49 millions per cu mm. The mean oxygen uptake per 10^9 cells per hour was 1.05 μl for men and 1.27 for women. This difference is statistically significant. The chance that the observed difference is due to random sampling error is only one in 1,744,000.

Serial determinations on one subject. (Table II). Because there appeared to be considerable variation in the oxygen uptake from donor to donor, the advisability of doing serial determinations on one donor became apparent. Over a 3-week period 12 samples of blood were obtained from a normal young man and each sample was measured in 6 manometers. Approximately 500 ml of blood were removed during this 21-day period. The red cell count tended to drop slightly during this period but there was no increase in reticulocyte count. The red cell count ranged between 5.06 millions and 4.11 millions per cu mm. The oxygen uptake varied from 0.8035 μl to 1.1913 μl per 10^9 cells per hour. These differences are statistically significant and indicate that the oxygen uptake of whole blood from the same donor varies from day to day.

Studies on preserved blood. Aliquots of blood stored as previously described were studied at frequent intervals from the time of collection until 64 days later. Serial determinations (Fig. 2) on each sample revealed that the oxygen uptake generally diminished about 50% during the first 20 days then fluctuated erratically throughout the remainder of the study. The pH of the blood varied from 6.6 to 7.0. Incubation in the Warburg flask for 2 hours did not alter the pH significantly. The oxygen uptake of stored blood was maintained at about 40% of the initial value from the 20th to 60th day of storage.

TABLE II. Fresh Blood with Sodium Citrate Collected from a Man at Various Times.

Date, 1949	μl $O_2/10^9$ cells/hr	Red blood cell count, $10^6/mm^3$
Oct. 22	.9061	4.85
24	.8685	5.06
25	.8929	4.61
26	.8035	4.98
27	.9288	4.66
28	.8752	4.68
31	1.0706	4.51
Nov. 1	1.0017	4.40
2	1.1913	4.32
3	.8411	4.11
7	1.0706	4.18
10	.8610	4.22

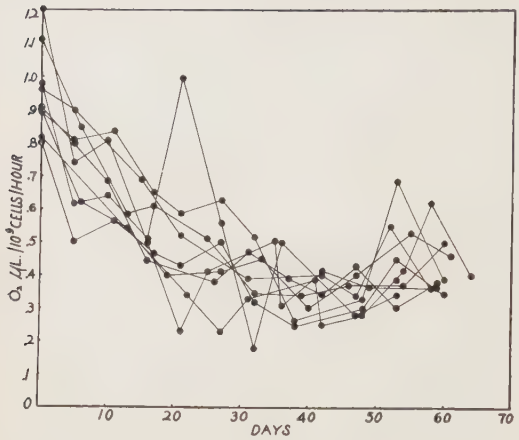

FIG. 2. Oxygen uptake of stored human erythrocytes. Each solid circle represents avg of 6 manometers. Connected solid circles indicate serial determinations on one flask of stored blood.

There was no evidence of gross contamination of the blood mixtures with bacteria. Contamination with bacteria does not account for the erratic oxygen uptake between the 20th and 60th days because an increased rate of oxygen uptake was not sustained. Furthermore in a 10-day period the oxygen uptake fluctuated from a low rate to high and back to the initial rate. These fluctuations are greater than the error of the technic of measurement. The explanation for a decrease cannot be due to progressive hemolysis of cells because the increases in the rate of oxygen uptake would not be explained. Likewise the decrease and erratic fluctuation of oxygen uptake cannot be explained by lysis of leucocytes. The fluctuations in the rate of oxygen uptake are very similar to the alterations in the dynamic state of stored erythrocytes observed(15) by Theile and Pennell. Their measurement of the metabolic state of the stored erythrocytes by estimation of evolved oxygen and cholinesterase activity showed comparable variability. No explanation is apparent.

Summary. The oxygen uptake of human whole blood is essentially the same whether sodium citrate or heparin is used as an anticoagulant. There is a significant difference between the oxygen uptake of the blood of men and women. A significant fluctuation was noted in the oxygen uptake of fresh blood from one person from day to day. The oxygen uptake of human blood stored in ACD solution rapidly decreases for 20 days then fluctuates for at least 60 more days in an erratic fashion.

The authors wish to acknowledge the help of Clinton D. Janney in the mathematical analysis of these data.

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Influence of Protoveratrine* on Effect of Vasoactive Drugs. (21405)

ANDRES GOTH AND FRANK HARRISON.

From the Departments of Pharmacology, Anatomy, and Pathology, Southwestern Medical School, University of Texas, Dallas.

The mechanism of the hypotensive effect of the veratrum alkaloids has been the subject of numerous investigations leading to varied and conflicting ideas as reviewed by Krayer and Acheson(1). As early as 1867, von Bezold and Hirt(2) suggested that these alkaloids owe their hypotensive action to sensitization of afferent mechanisms. The most recent investigations of Alexander *et al.*(3) and Gruhzit *et al.*(4) offer strong experimental support for von Bezold's theory. The latter group of investigators conclude that a mixture of veratrum alkaloids (veriloid) "causes hypotension by sensitization of stretch receptors, rather than by facilitation of central components of the reflex arc." While studying the mode of action of protoveratrine the observation was made that the blood pressure responses to vasoactive drugs were significantly enhanced following the injection of the veratrum alkaloid. Dogs and cats became more responsive to the hypertensive effects of 1-epinephrine and 1-nor-epinephrine as well as to the hypotensive influence of histamine and methacholine.

A study of the interaction of protoveratrine and vasoactive drugs was undertaken in an attempt to contribute to the understanding of the mechanisms involved. The problem was attacked by determining whether or not the neural actions of protoveratrine were necessary for eliciting the enhanced responses to 1-nor-epinephrine and histamine.

Methods. Two types of experimental preparations were utilized: (A) Animals with 9th and 10th nerves sectioned and (B) Animals with spinal cord transected at C6. The results reported in this paper were obtained on 6 dogs and one cat. A larger number of dogs and cats were used on preliminary and incomplete experiments. Anesthesia was obtained by the injection of 1 cc per kg of a solution containing 50 mg of chloralose and 500 mg of urethane. The solution was given intravenously to dogs and intraperitoneally to cats. Blood pressure was recorded by means of a mercury manometer from a cannulated femoral or carotid artery.

Results. Animals with 9th and 10th nerves sectioned. The 9th nerves were dissected bilaterally and sectioned close to the jugular foramen. The vagi were sectioned high in the neck. Repeated test doses of 1-nor-epinephrine and histamine were administered before and after the intravenous injection of protoveratrine (4 μg per kg).

Fig. 1 shows the typical response of a normal dog to 1-nor-epinephrine and histamine before and after protoveratrine. Fig. 2 shows the results in a dog whose 9th and 10th nerves were sectioned. As shown previously by Alexander *et al.*(3) and Gruhzit *et al.*(4), certain veratrum alkaloids fail to lower the blood pressure in dogs whose buffer nerves are sectioned; indeed they may produce a rise of pressure. It is clear from the experiment shown in Fig. 2 that the section of nerves 9 and 10 also prevented the usual influence of protoveratrine on the responses to 1-nor-epinephrine and histamine.

Animals with spinal cord transection at C6. The spinal cord was sectioned at the level of the sixth cervical vertebra. In addition both vagi were cut. After the blood pressure became stable repeated test doses of 1-nor-epinephrine and histamine were injected before and after the intravenous administration of protoveratrine. As shown in Fig. 3, protoveratrine had no effect on the blood pressure of cord transected animals. It also failed to modify the responses to 1-nor-epinephrine or histamine.

Discussion. The experiments reported in this paper confirm the recent investigations of

* The term protoveratrine refers to a mixture of Protoveratrines A and B supplied in commercial preparation Veralba. This drug was kindly given by Dr. Carl A. Bunde of Pitman-Moore, Indianapolis.

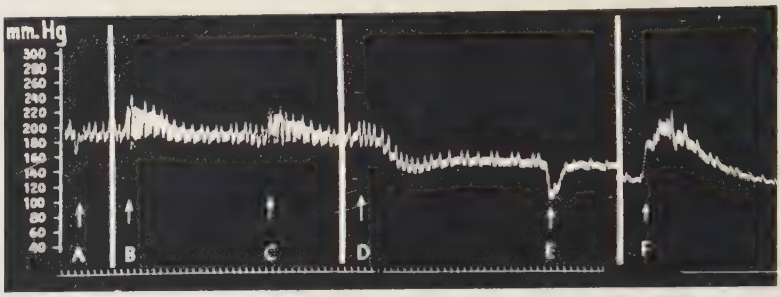

FIG. 1. Dog under chloralose-urethane anesthesia. Time in 15 sec. Changes of blood pressure induced by: A: 2 μg/kg histamine; B: 2 μg/kg l-nor-epinephrine; C: Repeat l-nor-epinephrine; D: 4 μg/kg protoveratrine; E: Repeat histamine; F: Repeat l-nor-epinephrine.

Gruhzit *et al.*(4), add a new fact and suggest a slightly different interpretation concerning the mechanism of the hypotensive action of protoveratrine. Since protoveratrine failed to lower the blood pressure of animals whose 9th and 10th nerves were sectioned bilaterally or whose spinal cord was transected it is obvious that its hypotensive action is not exerted directly on the cardiovascular system but is a consequence of a neural effect. The crucial experiments of Gruhzit *et al.*(4) established the fact that receptors along the distribution of the 9th and 10th nerves must be the site at which protoveratrine exerts its effect.

The findings of this paper indicate that protoveratrine enhances the responsiveness of dogs and cats to both pressor and depressor drugs. This effect also is a consequence of the neural action of protoveratrine since it does not occur in animals whose 9th and 10th nerves were cut or whose cord was transected.

Sensitization of baroceptors of the carotid sinus and those along the distribution of the vagus has been invoked by Gruhzit *et al.*(4) as the basic mode of action of certain Veratrum alkaloids including protoveratrine. If sensitization is interpreted as lowering of threshold of these receptors then the theory fails to explain our findings of enhancement of responses to pressor and depressor drugs unless the receptors have their threshold so lowered that they are maximally firing at the pressures encountered in these experiments.

In attributing to a drug a sensitizing action on a receptor one should state explicitly the intended meaning of "sensitization." Some workers mean by it an increased firing rate at some particular stimulus intensity. Others mean an increase in the slope of the stimulus-response curve, which may or may not imply an increased response at some particular stimulus strength. Others mean an increase in the rate of firing of the receptor irrespective of stimulus strength, and, perhaps even in the absence of a stimulus. In the latter case the slope of the stimulus-response curve may be decreased from the control period and may even approach horizontal or zero slope. It is in this latter sense that Jarisch *et al.*(5) use the word sensitization in giving the action of

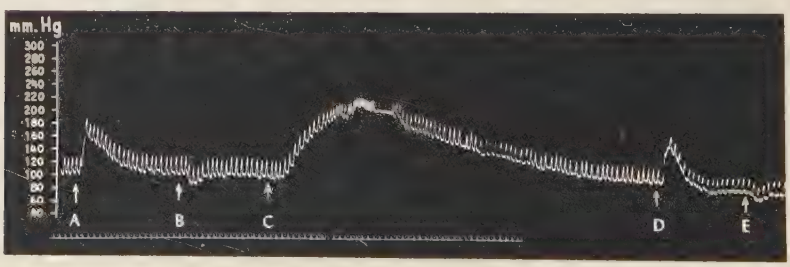

FIG. 2. Dog under chloralose-urethane anesthesia. Ninth and 10th nerves sectioned bilaterally. Changes of blood pressure induced by: A: .5 μg/kg l-nor-epinephrine; B: .5 μg/kg histamine; C: 4 μg/kg protoveratrine; D: Repeat l-nor-epinephrine; E: Repeat histamine.

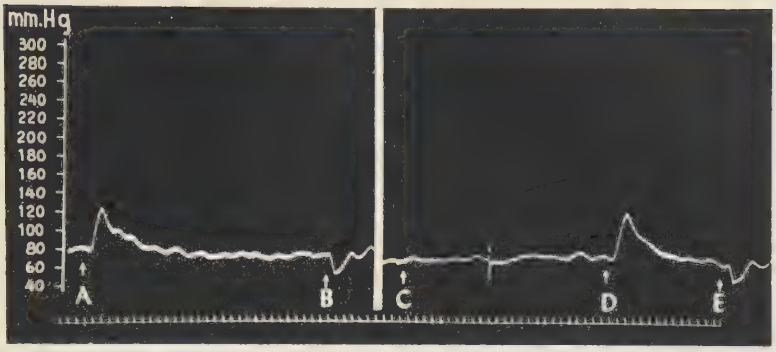

FIG. 3. Dog under chloralose-urethane anesthesia. Spinal cord and both vagi sectioned. Changes of blood pressure induced by: A: .25 μg/kg l-nor-epinephrine; B: .5 μg/kg histamine; C: 4 μg/kg protoveratrine; D: Repeat l-nor-epinephrine; E: Repeat histamine.

veratrine on carotid sinus receptors. By recording directly from the sinus nerve, they found that veratrine intraarterially at 10-40 μg levels caused a response from chemoceptors within a few seconds which disappeared within a few minutes, and a response from baroceptors which gradually increased to become almost a continuous discharge within 5 minutes. Witzleb(6) reported similar results. This latter action is what we believe to be present in our own experiments. We are preparing to test protoveratrine under direct recording from the sinus nerve to check these matters.

The following hypothesis would serve to harmonize the known facts concerning the mechanism of the hypotensive effect of protoveratrine: Protoveratrine causes spontaneous or repetitive firing of receptors of the carotid sinus and those along the distribution of the vagus. As a consequence of this, the blood pressure is lowered reflexly through inhibition of central vasomotor mechanisms. Because of the repetitive firing the baroceptors are less susceptible or non-susceptible to changes in pressure. This would lead to greater pressure changes following the injection of hypertensive or hypotensive drugs. This interpretation would fit in with the finding(7,8) that veratrum alkaloids may abolish the pressor response to carotid occlusion. Acheson and Rosenblueth(9) have demonstrated repetitive discharges of nerves induced by veratrum alkaloids (veratrine) in response to brief single stimuli.

Spontaneous or repetitive firing of carotid baroceptors induced by protoveratrine remains to be demonstrated. It is subject to experimental verification by recording action potentials from the sinus nerve.

Summary. Protoveratrine was found to enhance the blood pressure responses to 1-norepinephrine and histamine in dogs and cats. This effect failed to occur in animals whose 9th and 10th nerves were sectioned bilaterally or whose spinal cord was transected. It is suggested that the hypotensive effect of protoveratrine is due to induced spontaneous or repetitive firing of baroceptors, which then become less susceptible to further stimulation or inhibition by changes of pressure.

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Continuous Subcultivation of Epithelial-like Cells from Normal Human Tissues.* (21406)

R. Shih-man Chang.† (Introduced by John C. Snyder.)

From the Department of Microbiology, Harvard School of Public Health, Boston, Mass.

Epithelial-like cells have been successfully cultivated *in vitro* from various human tissues (1-3). Continuous subcultivation of epithelial-like cells derived from normal human tissue, however, has not been reported. The report of Swim and Parker(4) dealt with the subcultivation of fibroblasts and not epithelial cells from normal human tissue. The subject is worthy of further study, for, epithelial cells derived from certain human tissues may provide physico-chemical conditions suitable for the propagation of some viruses that have not been successfully propagated outside the human host.

In this report, a method for the serial subcultivation of epithelial-like cells derived from normal human tissues is described. By this method, epithelial-like cells from normal human conjunctiva, liver, kidney and appendix have been successfully subcultivated serially 30, 26, 22, and 16 times, respectively. The method is a modification of Gey's(5) for the subcultivation of epithelial-like cells derived from human uterine carcinomatous tissue. Epithelial-like cells are defined in this work as cells that are predominantly polyhydral with round or oval basophilic vesicular nuclei and abundant cytoplasm, grown on glass in single layers and in close apposition, to give the appearance of pavement epithelium.

Materials and method. The cultivation procedures, care of glassware and the preparation of media were in general similar to those used by other investigators(6,7). *Tissues:* All tissues were obtained surgically and were embedded in plasma clot by the roller tube technic within 2 hours. In cultivating the appendix, only the mucosa was used. *Human sera.* Human sera were obtained from several donors. The sera were inactivated at 56°C for 30 minutes and stored at 4°C. The sera were generally used up within 2 months after bleeding. Sera from various donors were not pooled for reasons to be discussed later. *Trypsin.* Stock trypsin solution was made by dissolving 0.5 g of "Difco" trypsin in 100 ml of balanced salt solution(6). The solution was sterilized by filtration through a Seitz filter. When kept frozen at –20°C, the solution was satisfactory for at least 3 months. Just before use, a 0.1% trypsin solution was prepared by diluting 1.5 ml of the stock solution in 0.5 ml of 1.4% $NaHCO_3$ and 5.5 ml of synthetic medium(6), subsequently referred to as MS. *Media.* The medium for the propagation of the cells consisted of 20% human serum, 5% chick embryo extract and 75% balanced salt solution. To 20 ml of this medium, 0.1 ml of 1% soya bean trypsin inhibitor and 0.1 ml of antibiotic solution, containing 1,000 units of penicillin and 1 mg of streptomycin were added. The medium was generally used up in 3 to 5 days. *Procedure for subcultivation.* The procedure outlined is for cells grown in roller tubes, 18 x 150 mm in size. For bottles and flasks, the volumes of trypsin and medium for resuspending cells are adjusted accordingly. We found that slight variation in these volumes did not influence the end results in any way. Treatment with trypsin consisted of the complete removal of the old medium; the addition of 2 ml of 0.1% trypsin per tube; the partial immersion of the tubes in a 37°C water bath for 5 to 10 minutes with frequent agitation to disperse the cell clumps; the complete removal of trypsin solution after centrifugation at 1000 rpm for 5 minutes; and, finally, the resuspension

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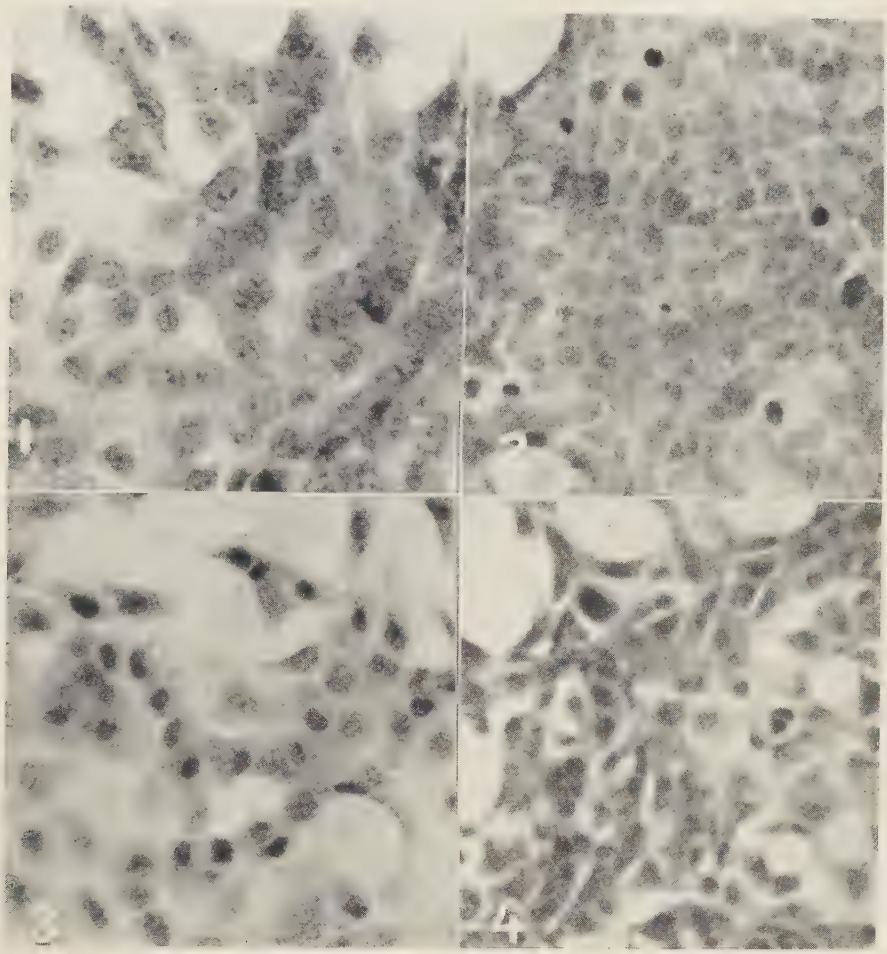

FIG. 1-4. Roller-tube cultures of human epithelial-like cells from 7th subculture of tissue from appendix (Fig. 1); from 15th subculture of tissue from liver (Fig. 2); from 20th subculture of tissue from conjunctiva; and, from 10th subculture of tissue from kidney (Fig. 4). Hematoxylin-eosin; 63×.

of the trypsinized cells in 1 ml of new medium. The trypsinized cells were distributed into 4 to 6 new tubes. One ml of medium was added to each tube, which was slanted in a 36°C incubator for 16 to 20 hours and then put in the roller drum at 36°C. The medium was completely changed on the 3d and 5th day after treatment with trypsin. Growth was usually sufficient for subcultivation in 5 to 7 days. During the earlier subcultures, when cell growth was apt to be irregular, the interval at which subcultivations could be made was best determined by the rapidity of growth. In the earlier subcultures, it was sometimes necessary to incubate tubes for as long as 30 days before undertaking subcultivation. In some instances the cells degenerated slowly. *Poliomyelitis virus.* Type 1 and 3 were obtained through the courtesy of Dr. H. A. Wenner of the National Foundation for Infantile Paralysis; type 2 was kindly furnished by Dr. J. F. Enders.

Results. With the method described, we succeeded in establishing 2 lines of epithelial-like cells from the conjunctivae of 4 patients. One of these lines was intentionally discontinued after the 16th subculture, while the other was subcultivated serially 30 times and is still being carried. We also succeeded in establishing epithelial-like cells from specimens of

kidney and liver. The kidney cells are currently at the 22d subculture and the liver cells in the 26th subculture. Of the 5 appendices, only one was successfully subcultivated and is now in the 16th subculture. Earlier attempts to subcultivate epithelial cells from human conjunctiva, liver, and kidney cultivated in media containing no human serum, such as, cow amniotic fluid medium(8) or horse serum embryo extract and balanced salt solution failed consistently. When the method of Swim and Parker(4) was used, fibroblasts completely overgrew the epithelial cells in the 2d to 4th subcultures. Fig. 1 to 4 show the subcultivated epithelial-like cells from the conjunctiva, liver, kidney and appendix. The morphological similarity of these cells was most striking. During the course of serial transfers it was not possible to differentiate between the cells from the four different tissues by studying fresh or cover-slip preparations stained with hematoxylin eosin or Giemsa. The difficulty of ascertaining the origin of cells growing *in vitro* is well established(9).

Importance of selecting blood donors. As the study progressed, it became apparent that serum from one donor might support the growth of one type of cell better than serum from another and that one serum might support the growth of one type of cell and yet be toxic to another type. We studied the sera from donors F, M, G, L and W. The conjunctival cells grew luxuriously in media made up with sera F and M; growth was slow in sera from G and W; while serum L was apparently toxic to the conjunctival cells. On the other hand, serum L supported the growth of the kidney cells best. The liver and appendix cells apparently grew best in serum G. It is therefore undesirable to pool sera from several donors. Some of the irregular results occurring in the earlier phases of this work may be traced to the use of serum from any donor. Difference of sera from different animals in supporting the growth of animal tissues *in vitro* had been described(9).

Maintenance of the cells in media containing no human serum. Because these established cells are no longer capable of propagation in media without human serum and because the presence of human serum in the medium may interfere with certain viral studies, experiments were conducted to select a suitable medium devoid of human serum to *maintain* the cells even though no further multiplication would occur. The media studied included 10% and 20% inactivated horse, ox, or rabbit sera in MS and in balanced salt solution; cow amniotic fluid medium(8); and human serum dialysate (prepared by dialyzing 50 ml of human serum in 200 ml of MS for 3 days at 4°C; the MS containing the dialyzable materials of the serum was sterilized by Seitz filtration). Five- to 7 day-old tubes with cells in good condition were selected; the fluid in each tube was completely replaced with 2 ml of the particular medium to be studied; the tubes were rolled at 36°C for 10 to 15 minutes; the fluids were again completely withdrawn and replaced with 2 ml of the same medium for each tube. The tubes were examined daily for 14 days. Media were changed every 3 to 4 days. Ten or 20% horse serum in MS consistently gave the best results. In this medium, all 4 types of cells were maintained in good condition for 10 to 14 days, after which varying degrees of degeneration took place. The cow amniotic fluid medium was equally suitable in the maintenance of the kidney cells. The human serum dialysate gave irregular results.

Susceptibility of conjunctival cells to 3 types of poliomyelitis virus. The 5th to 18th subcultures of conjunctival cells were used. Tubes with good growth were selected. Fluids were completely replaced with 2 ml of 10% inactivated horse serum in MS solution containing 50 units of penicillin and streptomycin (referred to as maintaining medium subsequently) for each tube. After the cultures had been rotated 10 to 15 minutes in the roller drum, the fluids were completely replaced with 1.35 ml of fresh maintaining medium to which 0.15 ml of stock virus, appropriately diluted, was added to each tube. The tubes were examined for degeneration daily, for 7 days. When the cells were completely degenerated, a portion of the medium was frozen at –70°C for subsequent titration and a portion was diluted to 10^{-3} for inoculation

of fresh tubes. All 3 types of poliomyelitis virus caused degeneration of the conjunctival cells in 2 - 5 days. Degeneration was prevented by type specific immune monkey serum. All 3 types of virus were passed serially 4 times with a theoretical increase in titer of 10^{12}. A comparative titration of one pool of type I poliomyelitis virus was made using the conjunctival, kidney and appendix cells. The ID_{50} were $10^{5.5}$, 10^{6}, and 10^{5} respectively.

Discussion. Through the use of a modification of Gey's technic(3,4), epithelial-like cells have been established and serially subcultivated from human conjunctiva, liver, kidney and appendix. Our method differs from Gey's in that the concentration of trypsin and time of exposure to trypsin are greatly reduced. The importance of the selection of donors for serum, on which the success or failure of establishing a cell line may largely depend, is stressed in this work.

The possibility that these epithelial-like cells may have arisen from extraneous tissue introduced in the medium was considered. The only possible source, in our opinion, was viable tissue that may have been present in the chick embryo extract. The 2 cycles of centrifugation at 1500 to 2000 rpm for 15 minutes and one cycle of slow freezing and thawing in the preparation of chick embryo extract rendered the presence of viable cells a very remote possibility. The fact that these epithelial-like cells grew only in medium containing human serum and not in cow amniotic fluid medium or medium containing horse serum, balanced salt solution and chick embryo extract, strongly suggests that these cells did not originate from chick tissue. The destructive effect of poliomyelitis virus on these cells indicated that they are of human and not of chick origin because chick tissue grown *in vitro* is not affected by poliomyelitis virus.

Summary. Epithelial-like cells from normal human conjunctiva, liver, kidney and appendix have been serially cultivated for 30, 26, 22, and 16 passages, respectively.

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Bound Iron and Unsaturated Iron-binding Capacity of Serum; Rapid and Reliable Quantitative Determination. (21407)

ARTHUR L. SCHADE, JIRO OYAMA, ROBERT W. REINHART, AND JOHN R. MILLER.

From the Laboratory of Infectious Diseases, National Microbiological Institute, National Institutes of Health, Bethesda, Md.

The total iron-binding capacity (TIBC) of serum and plasma is dependent upon the amount of siderophilin they contain(1-5). While it is possible to determine directly the siderophilin concentration by means of a specific precipitating antibody(3), this method is more involved and less informative than the indirect methods which establish the TIBC by summation of the fractions of iron-bound and iron-free siderophilin. The estimation of the siderophilin-bound iron is routinely made by one of a variety of methods recently reviewed and criticized by Hemmeler(6) and Laurell (7), [see also (8,9,10)]. Methods for the

determination of iron-free siderophilin in serum have been limited essentially to the direct iron-addition colorimetric method(1,11) and the indirect iron saturation excess methods (2,12,13,14,15).

The advantages of the proposed methods for the determination of bound serum iron and of iron-free siderophilin are: 1—analyses performed directly in serum or plasma sample without removal of constituent proteins or application of protein-denaturing heat and acid conditions; 2—use of a chromogenic iron-binding agent whose maximum spectral absorbency eliminates effectively interference by bilirubin; 3—use of a serum sample aliquot as its own control against interference by hemolysis products and other colored constituents of the serum; 4—each analysis can be run simply, rapidly, and reliably on 1 ml or less of serum.

Methods. Bound serum iron. The proposed method involves the adjustment of the serum or plasma sample by means of a concentrated phosphate buffer to a pH value at which the constituent proteins remain in solution but the siderophilin bound iron is wholly dissociated and available to combine with a suitable chromogenic reagent, in this case terpyridine(16). Ascorbic acid is used as the iron reductant. The iron-terpyridine complex in the treated serum is measured by the light absorption of a test solution at 552 mμ against a treated serum control minus terpyridine. A standard iron-terpyridine curve permits ready determination of the amount of iron in the original serum sample. *Reagents.* Phosphate buffer-ascorbic acid reagent. Molar sodium phosphate buffer, pH 5.3, was prepared from iron-free molar phosphoric acid and molar di-sodium phosphate solutions. To 100 ml of this buffer solution was added 1 g of ascorbic acid. The pH of the resulting solution was 5.0. Stock solution, kept refrigerated, is suitably stable for at least a week. 2, 2′, 2″-terpyridine reagent. A 0.10% solution was made by dissolving 100 mg of terpyridine in 4 ml of absolute ethanol and diluting to approximately 40 ml with water. The resulting milky suspension was cleared by the dropwise addition of 0.20 N HCl and then made to 100 ml volume. The

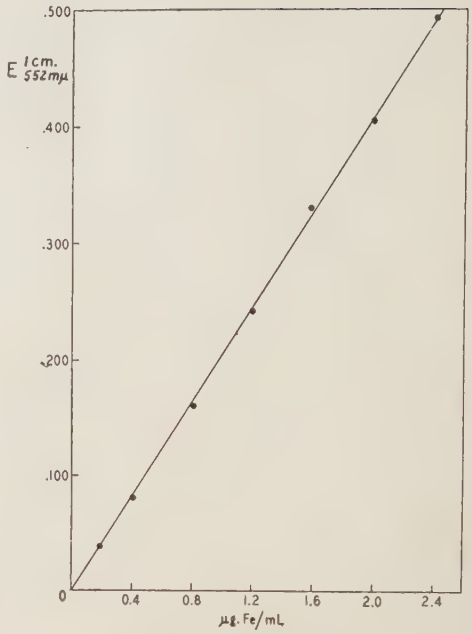

FIG. 1. Iron-terpyridine standard calibration curve as obtained under conditions of proposed bound iron and unsaturated iron-binding capacity (UIBC) tests.

final pH was 4.3. *Mixed reagents.* Reagent "A", prepared by mixing 4 parts of the phosphate buffer - ascorbic acid reagent with 6 parts of water, was used in the control serum sample. Reagent "B", prepared by mixing 4 parts of the phosphate buffer-ascorbic acid reagent with 2 parts of the terpyridine reagent, and 4 parts of water, was employed in the test serum sample. *Iron standard solutions.* A stock solution of iron containing 1 mg/ml was prepared by dissolving electrolytic iron in a minimal volume of 10 N sulfuric acid followed by dilution with water. Appropriate dilutions of the stock solution were made with 0.01 N hydrochloric acid. From such dilutions, a standard iron-terpyridine curve was established under the conditions of the test, (Fig. 1). *Iron-free water* was used in the preparation of the reagents. All glassware was soaked overnight in nitric acid diluted 1:1 with water and then rinsed repeatedly with iron-free water.

Procedure. 0.5 ml of serum or heparinized plasma (0.1 mg per ml of blood) are pipetted into 2 test tubes. To one tube are added 0.5 ml of mixed reagent A and to the other, 0.5

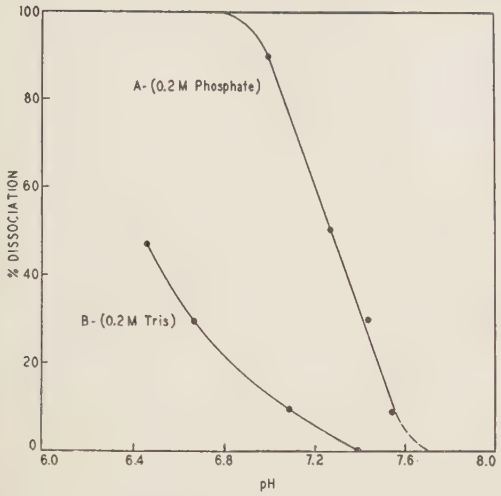

FIG. 2. pH dissociation curves of heparinized plasma iron-siderophilin (95% saturated) derived from iron-terpyridine complex as determined by bound iron method (curve A) and by UIBC method (curve B).

ml of mixed reagent B. The final pH in both tubes is approximately 6.0. Both tubes are then placed in a 45°C water bath for 20 minutes. After this period, a sample of each tube is transferred to 0.5 ml volume cuvettes of 1 cm light path and read in a Beckman spectrophotometer at 552 mμ. The difference in the extinction values, corrected for reagent blank (0.5 ml water plus 0.5 ml of mixed reagent B versus 0.5 ml water plus 0.5 ml of mixed reagent A), gives the amount of bound iron in the 0.5 ml of test serum when read off the standard iron-terpyridine curve.

Discussion. The simplification introduced by the present method of analysis of the bound iron of serum is the use of serum directly, without precipitation of its proteins, to serve as its own control against an identical sample plus the iron chromogenic agent, terpyridine. Of particular importance in making this possible is the relatively high pH, (6.0) at which the test is run. The high final concentration of phosphate buffer (0.2M) at this pH effects complete dissociation of the iron from its bound state in serum and makes it available for complexing with terpyridine. Fig. 2, curve A, illustrates as a function of pH the dissociation of the iron from siderophilin in a sample of heparinized plasma, made 0.2 M with respect to phosphate buffer, to which sufficient iron was added to make the siderophilin 95% iron-saturated. From examination of this curve, it is apparent that the siderophilin-bound iron is completely dissociated under the conditions of the test.

To determine whether differences in the concentrations of hemoglobin or bilirubin in the samples of serum tested affected the siderophilin-bound iron values, analyses were run on sera to which these substances were added. As much as 250 μg crystalline human hemoglobin† added to a milliliter of serum, or roughly 200% that found in what is considered "hemolyzed" serum, had no effect on the bound iron determination. Analysis of a hemoglobin solution containing the equivalent of 2.4 μg iron/ml showed no iron under conditions of the test. Additions of red cell lysate to serum samples were also without effect. When sufficient bilirubin (10 μg/ml) was added to serum to give it an icteric index value of 30, [normal = 4 to 6; latent jaundice = 6 to 15 (17)], no interference with the bound iron analysis of the serum was observed. A clinical serum sample containing 24 mg bilirubin per cent was also satisfactorily analyzed. Further, when known amounts of iron were added to serum to give a range of 1 to 4 μg of iron per ml of serum their recoveries were complete. These results add confirmation to the view that the presence of serum constituents does not interfere with the determination of siderophilin-bound iron by the present method.

For comparative purposes, 6 plasma samples chosen at random from normal as well as pathological donors were analyzed by the proposed method and by Ramsey's method for serum iron(9), substituting in the latter method terpyridine for 1, 1′-dipyridine. The results are summarized in Table I. Considering the relatively limited sensitivity of the Ramsey method which results from the greater dilution of the original serum sample, the iron analyses by both methods are in substantial agreement.

Unsaturated iron-binding capacity (UIBC) of serum. The proposed method involves the

† Courtesy of Dr. William H. Crosby, M.D., U. S. Army, Walter Reed Hospital, Washington, D. C.

TABLE I. Comparative Analyses of Serum Iron by Ramsey's Method and by the Proposed Method.

Serum sample	μg Fe % (Ramsey)	μg Fe % (Schade *et al.*)
1	50	56
2	14	21
3	155	141
4	18	26
5	60	68
6	80	75

determination of the unsaturated siderophilin by addition to serum of iron in excess of that capable of being bound, followed by direct analysis of the serum sample with terpyridine as the chromogenic agent without acidification or the removal of the constituent proteins. A serum control to which the same amount of iron but no terpyridine is added furnishes the necessary correction for the absorption at 552 mμ of the iron-siderophilin complex in the test sample(1). The difference between the quantity of iron added to the serum and that found to be in excess is equal to the UIBC. *Reagents.* "Tris" buffer reagent. Molar "tris" buffer, [tris-(hydroxymethyl)-aminomethane], adjusted to pH 8.5 with hydrochloric acid, was prepared. 2, 2′, 2″-terpyridine reagent. (See under bound iron method). "Elon" reagent. A 1% "Elon" (p-methylaminophenol sulfate) solution was prepared and stabilized with 3% sodium bisulfite in water(18). Iron standard solutions. (See under bound iron method).

Procedure. To 0.5 ml serum or heparinized plasma in each of two tubes are added 0.2 ml of the "tris" buffer reagent and 0.1 ml of a standard iron solution (usually 25 μg Fe/ml). The tubes are capped and placed in a 45°C bath for 10 minutes to allow development of the iron-siderophilin complex. At the end of this period, to the control tube are added 0.1 ml of water and 0.1 ml of the "Elon" reagent. To the test tube are added 0.1 ml of the terpyridine reagent and 0.1 ml of the "Elon" reagent. The final volume is 1 ml. Both tubes are recapped and placed in the 45°C bath for 30 minutes. The test sample is then read against the control at 552 mμ in a Beckman spectrophotometer as described above for the bound iron procedure. The difference in the extinction values, when corrected for a reagent blank composed of 0.6 ml of water, 0.2 ml of "tris" buffer, 0.1 ml terpyridine reagent, and 0.1 ml "Elon" reagent versus an identical mixture with 0.1 ml water substituted for terpyridine, gives the amount of excess iron when read off the standard iron-terpyridine curve. Subtraction of the determined amount of excess iron from the known amount added yields the μg of iron bound per 0.5 ml of serum.

Discussion. The final pH of the serum after addition of "tris" buffer and the other reagents was approximately 8.3. This value was sufficiently high to assure 100% association of the siderophilin-bound iron in serum or in plasma treated with heparin (Fig. 2, curve B). Thus, there was no interference by the bound iron with the determination of that added in excess. Further, in cases where the plasma sample had been obtained from blood-bank A.C.D.-treated blood, (1.84 g dextrose, 1.65 g sodium citrate, 0.6 g citric acid in 75 ml added to 500 ml of blood), these test conditions were suitable for UIBC determinations despite the fact that the iron-siderophilin dissociation curve was significantly shifted to the alkaline side by the presence of an approximately 0.027 M citrate concentration. For example, while the dissociation of the iron-siderophilin complex in heparinized plasma in the presence of 0.2 molar "tris" buffer was evident only below pH 7.4, dissociation in 0.2 molar "tris" buffer plus 0.027 molar citrate began at pH 8.0.

The choice of 10 minutes as an adequate incubation period for the added iron to complex with the unsaturated siderophilin of the serum sample was decided upon after investigation showed that 90% of saturation was accomplished after 2.5 minutes and 100% by the end of a 5 minute period at 45°C. It may be noted that with human serum or plasma samples, very little or none of the excess iron added as ferrous is detectable by terpyridine in the absence of "Elon" by the end of the 10 minute incubation period. The addition of 5 μg of iron per ml of serum, to exceed the serum's binding capacity, was generally suitable for the determination of the UIBC of most pathological as well as normal sera, *i.e.*, UIBC values ranging from 100 to 400 μg Fe %. Where the UIBC fell below 100 μg Fe %,

3 μg of iron per ml of serum (0.1 ml of a standard iron solution containing 15 μg Fe/ml for each 0.5 ml of serum) were employed. *"Elon"* proved to be a satisfactory, iron-free, reducing agent. The iron-terpyridine standard curve obtained with its use at the pH of the test, pH 8.3, was the same as that found with ascorbic acid at pH 6.0 as employed in the bound iron analysis. Hydrosulfite could also be used for the UIBC determinations but its stability as a stock solution was much inferior to that of "Elon." The employment of an aliquot of the serum sample plus the same amount of added iron and reducing agent but minus terpyridine provided good control for the various colored serum constituents and assured that the absorption at 552 mμ was due solely to the iron-terpyridine complex. As in the bound-iron analysis, no interference from bilirubin, hemoglobin, or the red cell lysate concentration in the serum sample was observed. The iron-siderophilin complex, whose amount in any given serum sample depends directly on its total iron-binding capacity, effects significant absorption of light at 552 mμ, ca. 40% of its absorption maximum at 460 mμ, (19). The control serum sample with its added iron in excess afforded self-correction for the absorption at 552 mμ resulting from the formation of this complex in the test sample.

For comparison of the UIBC method just described with the direct method(1) in which increasing amounts of iron are added to a serum sample and the absorptions at 465 mμ are recorded, a suitable, practically colorless plasma sample with a high UIBC was employed to obtain the UIBC by both methods. Fig. 3 summarizes the results of the comparison. Curve A shows a sharp inflection at approximately the point of 2 μg of iron added to the 0.5 ml of plasma used, while curve B likewise illustrates saturation of the siderophilin of the plasma sample upon addition of the same amount of added iron. Fig. 3, curve A, further shows that no terpyridine-iron color whatever becomes evident under the conditions of the test until saturation has been achieved.

The increase in sensitivity of the proposed method over the direct method is evident from inspection of the slopes of curves A and B in Fig. 3. In the direct method, the addition of

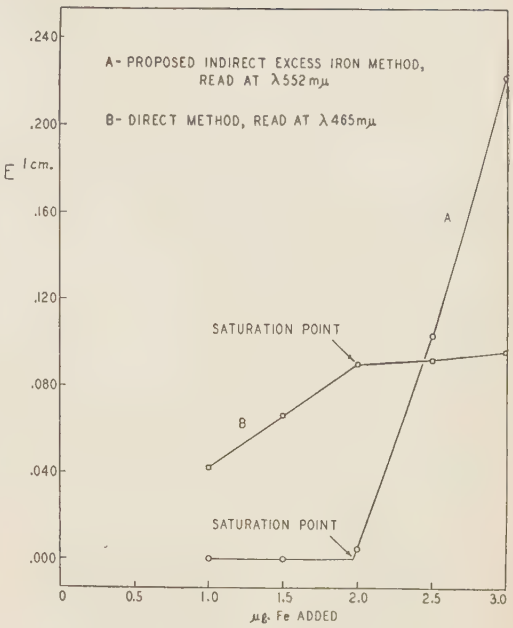

FIG. 3. UIBC of the same heparinized plasma sample as determined both by direct method and by proposed method. Saturation point of curve A represents point beyond which added iron, now in excess, is determinable as iron-terpyridine. Saturation point of curve B is point beyond which added iron, now in excess, no longer is determinable as the iron-siderophilin complex by absorption at 465 mμ.

1 μg of iron to the plasma sample effects an optical density change of 0.046 at the maximum absorption wavelength for iron-siderophilin of 465 mμ up to the saturation point. In the proposed method, the addition of 1 μg of iron gives an optical density change of 0.200 at the maximum absorption wavelength for iron-terpyridine of 552 mμ beyond the saturation point. Thus, greater than a 4 fold increase in sensitivity is obtained by the proposed method.

Greater sensitivity in the determinations of both the bound iron and the UIBC can be readily obtained by decreasing the amount of dilution of the original plasma sample provided that the concentrations of the reagents in the final volume of the test sample are equivalent to those here recommended. Further, if suitably large amounts of serum or plasma are available for analysis, the conventional Beckman absorption cells 1 cm x 1 cm x 4 cm can be employed as well as test

tubes for use in colorimeters provided with a satisfactory filter for 552 mμ.

Preliminary investigations with 4, 7-diphenyl-1, 10-phenanthroline‡, whose molar absorbency index is approximately twice that of terpyridine, have shown that this reagent can be substituted for terpyridine, with minor modifications, in both the bound iron and UIBC analytical methods. While 4, 7-diphenyl-1, 10-phenanthroline is quite insoluble in aqueous buffer solutions, it rapidly becomes soluble in plasma or serum at 45°C under conditions of the iron tests and thus can be employed to give increased sensitivity where necessary.

Summary. 1. New, rapid, and reliable methods for determination of siderophilin-bound iron and of iron-free siderophilin in small amounts of sera or plasma have been devised. 2. Bound serum iron is determined by adjustment of serum sample with concentrated phosphate buffer to a pH value at which constituent proteins remain in solution but siderophilin-bound iron is wholly dissociated and available to combine with a suitable chromogenic agent, (terpyridine). Ascorbic acid is used as the iron reductant. Iron-terpyridine complex in treated serum is measured by light absorption of a test solution at 552 mμ against a treated serum control minus terpyridine. A standard iron-terpyridine curve permits ready estimation of amount of iron in original serum sample. 3. The proposed method for determination of iron-free siderophilin, or unsaturated iron-binding capacity of serum (UIBC), involves addition to serum of iron in excess of that capable of being bound, followed by direct analysis of serum sample with terpyridine as the chromogenic agent without acidification or the removal of constituent proteins. A serum control to which the same amount of iron but no terpyridine is added furnishes the necessary correction for the absorption at 552 mμ of iron-siderophilin complex in the test sample. The difference between quantity of iron added to serum and that found to be in excess is equal to the UIBC.

‡ Both terpyridine and this chromogenic reagent are obtainable from G. Frederick Smith Chemical Co., Columbus, O.

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Hypnotic Action Resulting from Combined Administration of Salicylamide and Acetophenetidin. (21408)

F. M. Berger.*

From the Wallace Laboratories, Division of Carter Products Inc., New Brunswick, N. J.

Acetophenetidin (phenacetin) and salicylamide have been used for many years as mild analgesics. The present paper describes the unsuspected hypnotic effect obtained when these 2 drugs are administered together in suitable proportions.

Methods. The hypnotic and lethal actions of the drugs were determined in mice after intraperitoneal administration in 5% aqueous gum acacia. Loss of the righting reflex was taken as the criterion of hypnosis. Joint action of the 2 drugs was evaluated by a technic similar to that proposed by Bliss(1,2). Mixtures of salicylamide and acetophenetidin were prepared in proportions of 1:3, 2:2, and 3:1, in fractions of their median hypnotic doses (HD_{50}) or median lethal doses (LD_{50}). Groups of 20 mice were injected with graded doses of each mixture and the HD_{50}, the LD_{50}, the surely hypnotic dose (HD_{99}) and the usually safe dose (LD_1) were found graphically by Miller and Tainter's method(3).

Results. Table I indicates that salicylamide has a hypnotic action and possesses an appreciable margin between the HD_{50} and the LD_{50}. However, it does not have any standard safety margin (SSM) as defined by Foster (4) because the dose that produces sleep in all animals (HD_{99}) is greater than the largest amount tolerated without death (LD_1). Acetophenetidin has no true hypnotic action. The drug produces loss of righting reflex only in doses that will kill some animals. When the two were given in combination, true hypnotic action was found. Mixtures of 3 parts of salicylamide to 1 of acetophenetidin and mixtures with equal amounts of the two possessed a wider margin between the HD_{50} and LD_{50} than either of the ingredients given separately. These mixtures also had an appreciable safety margin between surely effective and largest safe doses. Mixtures of 3 parts acetophenetidin to 1 part salicylamide had a narrower margin between the HD_{50} and LD_{50}, and a smaller standard safety margin than the other mixtures.

The nature of synergism between the drugs is shown in Fig. 1. The HD_{50} or LD_{50} of the various mixtures are plotted in terms of salicylamide against the concentration of acetophenetidin in the mixture. The line OA represents the HD_{50} or LD_{50} in the absence of acetophenetidin and the line OB, the HD_{50} or LD_{50} of acetophenetidin alone. If the drugs have a simple additive action, this joint effect will be represented by points on the line AB. Effects shown by points inside the triangle AOB signify potentiation, while points above the line AB signify incomplete addition.

When the effect of the combinations was judged by their ability to produce hypnosis, the synergism observed depended to a marked extent on the proportion of the 2 ingredients in the mixture. Thus mixtures of 3 parts salicylamide to 1 part acetophenetidin showed marked potentiation while mixtures of 1 part salicylamide to 3 parts acetophenetidin approached simple additive action.

Administration of lethal doses of the various mixtures always produced incompletely additive effects. The effectiveness of the mixtures appeared to be independent of the ratio of the 2 components.

The duration of sleep after administration of various mixtures of salicylamide and acetophenetidin is given in Table II. The joint action of the drugs in doses which by themselves were not somnifacient, produced sleep lasting over 2½ hours. Reducing the dose of acetophenetidin in the mixture while keeping the amount of salicylamide constant had less effect on the duration of sleep than a reduction of the amount of salicylamide.

Experiments were also carried out to determine which compounds chemically related to salicylamide would produce sleep when ad-

* I am obliged to Mr. Thomas E. Lynes and Mrs. P. Purvis for technical assistance.

TABLE I. Median hypnotic (HD_{50}), Median Lethal (LD_{50}), Certainly Hypnotic (HD_{99}) and Usually Safe (LD_1) Doses of Salicylamide, Acetophenetidin and Mixtures of the 2 Drugs Prepared in Fractions of Their HD_{50} and LD_{50} Doses. Mice, intraperitoneal administration. Effectiveness of mixtures expressed in terms of their salicylamide contents. All doses in mg/kg.

	Salicylamide	Acetophenetidin	Mixtures of salicylamide and acetophenetidin in proportion of 3:1	1:1	1:3
HD_{50}	370 ± 18	655 ± 36	195 ± 7	143 ± 4	96 ± 3
HD_{99}	760	1065	247	184	130
LD_{50}	860 ± 60	820 ± 66	670 ± 41	500 ± 34	257 ± 12
LD_1	360	295	410	305	158
$LD_{50} : HD_{50}$	2.3	1.3	3.4	3.5	2.7
SSM*	0	0	66%	66%	21%

* Standard safety margin $\left(\frac{LD_1}{HD_{99}} - 1\right) 100$.

ministered in combination with acetophenetidin. In these experiments, groups of 10 mice received acetophenetidin 280 mg/kg together with 280 mg/kg of the compound to be tested. Acetophenetidin and the other compounds alone did not produce hypnosis at this dose.

Of the compounds tested only benzamide and benzene-sulfonamide produced hypnosis when given jointly with acetophenetidin. Benzamide was more effective than benzenesulfonamide but not as good as salicylamide. *m*-Hydroxybenzamide and *p*-hydroxybenzamide, the isomers of salicylamide were ineffective, as were acetamide, *o*-phthalamide, 2-furamide, nicotinamide, gentisamide, salicylic acid, gentisic acid, acetylsalicylic acid, phenyl salicylate, *p*-aminobenzoic acid, *p*-acetaminophenyl salicylate, antipyrine, aminopyrine, and pyribenzamine.

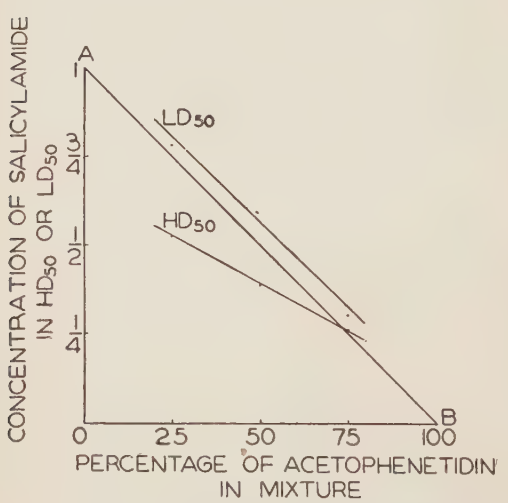

FIG. 1. Mean effective and mean lethal doses of mixtures of salicylamide with acetophenetidin plotted in terms of salicylamide against concentrations of acetophenetidin in mixtures.

In other trials various antipyretics and analgesics related to acetophenetidin were injected in combination with salicylamide 280 mg/kg. (This dose of salicylamide given by itself did not produce loss of righting reflex.) Antipyrine, aminopyrine, p-aminophenol in doses of 280 mg/kg and acetanilid at 180 mg/kg did not produce loss of the righting reflex. When given jointly with salicylamide, hypnosis was obtained. The duration of sleep varied with the compound used and was usually shorter than that obtained after combined administration of acetophenetidin and salicylamide.

Discussion. The central depressant action of salicylamide was described by Nebelthau in 1895(5) and has more recently been confirmed by Hart(6) and by Litter *et al.*(7). Salicylamide, however, has not been used as a hypnotic and would not be suitable for this purpose because of its weak action and the narrow margin between lethal and hypnotic doses. References relating to a hypnotic action of phenacetin have not been found in the literature.

Our results show that acetophenetidin will potentiate the hypnotic action of salicylamide. At the same time potentiation of the toxic properties of these drugs will not occur after their joint administration. The different extent of joint action occurring after adminis-

TABLE II. Duration of Sleep in Mice after Intraperitoneal Administration of Various Mixtures of Salicylamide and Acetophenetidin. Ten mice per dose.

Salicylamide, mg/kg	Acetophenetidin, mg/kg	% of animals asleep	Duration in min. ± SE
280	0	0	0
0	420	0	0
280	420	100	154 ± 9
280	280	100	119 ± 10
280	180	100	59 ± 5
280	120	100	70 ± 12
280	80	70	65 ± 8
280	55	0	0
180	420	100	67 ± 9
120	420	40	31 ± 8
80	420	0	0

tration of the 2 drugs in hypnotic and toxic doses causes a substantial widening of the therapeutic index and suggest a possible usefulness of such combinations as sedatives and hypnotics.

Summary. Ineffective doses of salicylamide and acetophenetidin when administered in combination produce hypnosis of considerable duration. An analysis of the synergism between the 2 drugs indicates potentiation when the drugs are administered in terms of their median doses causing loss of the righting reflex. When the 2 drugs are given in terms of their median lethal doses an incompletely additive effect is obtained. The ability of related drugs to replace salicylamide or acetophenetidin in the mixtures was also investigated.

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Excretion of a Histidine Metabolite by the Rat. (21409)

MILTON SILVERMAN, HOWARD A. BAKERMAN, AND FLOYD S. DAFT.

From the National Institute of Arthritis and Metabolic Diseases, National Institutes of Health, Public Health Service, U. S. Department of Health, Education, and Welfare, Bethesda, Md.

It has been reported that in folic acid deficiency, the rat excretes a heat and alkali labile compound which contains glutamic acid and which is derived from histidine(1-3). This compound is identical with one that accumulates during the metabolism of histidine by liver preparations(4-6). Hydrolysis by acid or alkali of this glutamate derivative gives rise to a mole each of L-glutamic acid, formic acid and ammonia. This derivative has been postulated to be α-formamidinoglutaric acid (4,7) and will be referred to in this report as FAG. Since under normal dietary conditions this material does not appear in the urine of the rat, it was assumed that the compound was readily metabolized by the animal which was not folic acid deficient. However, FAG, when injected subcutaneously, is excreted almost completely by both the normal and folic acid deficient rat. This report deals with the excretion of the metabolite, derived from histidine, by control and folic deficient rats.

Methods. The FAG employed for injection was isolated from the urine of folic deficient rats by a method described previously(2). The procedure was modified by the rejection of impurities in the second Dowex 50 eluates which, at pH 2.0, were insoluble in 5 volumes of ethanol. The material was obtained in the form of the amorphous barium salt and converted into the sodium salt (by reaction with sodium sulfate) before use. The samples employed for injection were 95% pure based on their "labile glutamic acid" content. The

TABLE I. Recovery of "Labile Glutamic Acid" Added to Urine.

	γ glutamic acid activity/ml		γ "labile glutamic acid"/ml		
	A Heated	B Unheated	2A-B	Difference	Recovery, %
1. 960 γ of "labile glutamic acid"/ml	484	40	928		97
2. Urine from folic deficient rat	340	84	596		
3. Sample 2 containing 960 γ of "labile glutamic acid"/ml	810	124	1496	900	94
4. Urine from control rat	74	124	24		
5. Sample 4 containing 960 γ of "labile glutamic acid"/ml	540	142	938	914	95

Recoveries based on 960 γ "labile glutamic acid" present in Sample 1.

contaminating material consisted essentially of L-glutamic acid. "Labile glutamic acid" in this material was determined by the increase in "free" glutamic acid activity of samples after storage in 0.5 N-KOH for 24 hours at 37°C. "Labile glutamic acid" as employed in this report refers to glutamic acid activity which can be derived from the heat or alkali degradation of the FAG. The analytical procedures were those described previously(1,2). For the determination of "heat labile glutamic acid", samples were autoclaved for 10 minutes at 10 lb steam pressure. This process makes approximately 50% of the glutamic acid contained in the FAG available to *Lactobacillus arabinosus*. Filter sterilization of the samples was employed in the determination of "free" glutamic acid. "Free" glutamic acid includes all of those forms of glutamic acid which are available for growth of *L. arabinosus*. The type of rat, criteria for folic acid deficiency, maintenance of animals, diets, and collection of urine specimens were the same as previously described(1) except that the diet of the control animals was supplemented with 20 γ of folic acid per g of diet.

Experimental. The analytical procedure employed to determine the concentration of the FAG is based on the heat degradation of the material to a compound having glutamic acid growth activity for *L. arabinosus*. The application of alkaline hydrolysis to the determination of FAG in urine was found to be less reliable than the use of heat degradation. Glutamic acid growth activity values were found for A) samples added to the basal medium at pH 6.2 and then heat sterilized, and B) samples sterilized by filtration (unheated) and added aseptically to the heat sterilized basal medium. Recoveries satisfactory for the

TABLE II. Excretion of "Labile Glutamic Acid" after Subcutaneous Injection.

	mg glutamic acid activity excreted/day		mg "labile glutamic acid" excreted		
Rat	A Heated	B Unheated	Per day 2A-B	Excess over control period	Recovery, %
Folic deficient #19131					
Control period	9.0	2.6	15.4		
Exp. "	18.4	2.2	34.6	19.2	77
Folic deficient #19146					
Control period	8.6	2.0	15.2		
Exp. "	21.5	2.2	40.8	25.6	102
Control #19135					
Control period	1.4	2.2	.6		
Exp. "	14.6	3.4	25.8	25.2	101
Control #19145					
Control period	2.0	3.8	.2		
Exp. "	14.1	3.0	25.2	25.0	100

Control period represents a 3-day collection of urine prior to first injection of "labile glutamate." During experimental period 25 mg of "labile glutamic acid" were injected each day for 3 days. A 3-day collection of urine was started immediately after first injection.

TABLE III. Excretion of "Free" Histidine after Subcutaneous Injection.

Urine collection No.		1		2	3
1	Control (A651)	360 γ (0)	50 mg of L-histidine inj.	210 γ (0)	130 γ (0)
2	" (A653)	290 γ (0)		290 γ (13)	270 γ (0)
3	Folic def. (A1050)	170 γ (22)		480 γ (64)	370 γ (31)
4	" " (A1137)	100 γ (2.0)		230 γ (35)	110 γ (10)

Values indicate daily excretion of "free" histidine. Values in parentheses indicate mg "labile glutamate." Each collection represents a consecutive 24 hr period.

present purpose were obtained by subtracting the value found for B from twice that of A. The results are reported in terms of "labile glutamic acid" derived from FAG.

Table I gives an indication of the recoveries obtained. The sample added to the urine specimens contained 4% glutamic acid in the "free" form and 96% in the labile "fixed" form. Using the calculations indicated, recoveries in the order of 95% of the material added to urine can be obtained. The method is satisfactory as a quantitative procedure only when the bulk of the glutamic acid activity originates from FAG. The procedure cannot validly be applied to specimens containing relatively high concentrations of glutamine (human urine). It must be emphasized the method is empirical and is not recommended for general application.

Two control and 2 folic acid deficient rats were selected to receive subcutaneous injections of the labile glutamate compound. A 3-day urine collection was obtained from each prior to the injections. The rats were then given subcutaneously 25 mg of the labile form of glutamic acid, each day for 3 days. The urine which each rat excreted in this 3-day period was analyzed for the labile glutamate compound. The bulk of the injected derivative was found in the urine of each of the rats (Table II).

These results were not expected in the case of the control animals. Both control and folic acid deficient animals readily utilized a 50-mg dose of either histidine (Table III) or glutamic acid (Table IV). More than 99% of the histidine had been utilized and from 70 to 90% of the glutamic acid had been metabolized. It is of some interest that one of the control rats given 50 mg of histidine, excreted 13 mg of glutamic acid in a heat labile form. In any case such rats seem to be able to metabolize histidine and glutamic acid, an end product of histidine metabolism.

Discussion. The reason for the inability of the normal rat to utilize this FAG when injected is not evident. It has been suggested the lack of the utilization of this histidine in-

TABLE IV. Excretion of "Free" Glutamic Acid after Subcutaneous Injection.

Collection No. / Rat No.	1		2		3	% of inj. glutamic acid excreted
	"Free" glutamic acid excreted/day, mg					
Folic deficient		50 mg glutamic acid inj./day		Inj. discontinued		
19139	1.2		10.2		Died	18
19149	1.2		6.8		"	11
19111	4.3		12.3		"	16
19115	3.9		9.8		2.9	12
19116	4.3		21.0		Died	33
19153	1.8		11.4		2.0	19
Control						
19135	2.4		6.8		2.2	9
19145	3.3		7.9		3.8	9
19140	3.5		6.0		3.2	5
19150	4.6		17.3		10.9	25

Each collection represents a consecutive 72-hr period. During collection period No. 2, 50 mg of L-glutamic acid injected each day.

termediate by liver preparations may be due to the absence of an acceptor for the potential formyl group or destruction of a necessary enzymic component(4). In the intact normal rat, however, it would not be expected that the necessary enzymatic mechanisms were lacking. The possibility does exist that the rate of formation of a suitable formyl acceptor (imidazole-carboxamide-ribotide ?(8)) is insufficient to accommodate a single large dose of a direct formyl donor. Thus the bulk of the material was excreted before it was metabolized. Alternative explanations for the lack of utilization are that FAG enters the cells involved in its metabolism at insignificant rates or that the labile compound is not in the pathway of the normal metabolism of histidine despite its excretion by the folic acid deficient rat.

Summary. A histidine metabolite, isolated from the urine of folic acid deficient rats and postulated to be α-formamidinoglutaric acid, is excreted unchanged after subcutaneous injection into normal rats.

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Total Body Water in Rats and in Mice. (21410)

JOHN ANNEGERS.

From Naval Medical Research Institute, Bethesda, Md.

One of the assumptions that is made in the estimation of body fat from measurements of total body water(1-4) is that the fat-free body has a "constant" fraction of water. This assumption is supported by measurements of body water by desiccation and body fat by ether extraction in normal rats(5) and in eviscerated, normal guinea pigs(6), since in both studies total body water was nearly a direct proportion of fat-free body weight. On the other hand, this relationship appears to differ between normal rats in a neutral environment and those subjected to cold(7), and between weanling and grown rats(8). Furthermore, it has been pointed out(9) that in the data from guinea pigs(6) the percent water in the fat-free body is positively correlated with the percent body fat. However, when this method of analyzing the data, in which the two variables fat and fat-free weight may be confounded, is applied to additional data in the literature, the percent water may appear to increase(10) or to decrease(5) with the percent body fat.

Accordingly, the present study was undertaken in order to reinvestigate the relationships among the 3 variables of total body water, body fat, and fat-free, dry body weight in normal rats and mice and in starved and dehydrated rats.

Methods. Animals were killed by a blow on the neck, clipped, and weighed. The mice were cut into small pieces with scissors and frozen in liquid nitrogen. The entire rats were run through a hand-operated meat grinder 3 times, washed into a Waring blender with warm water, weighed, and duplicate aliquots were weighed and frozen in liquid nitrogen. The frozen tissues were placed in a vacuum desiccator at room temperature for 24 hours, and for an additional 48 hours at 50°C, after which the dry weights were taken. Total body water was calculated from the dead weight, blended weight, wet aliquot weight, and dry

aliquot weight. This method for total body water was chosen upon finding that the tissues lost no more weight after 72 hours of vacuum drying and that the water contents checked with those obtained on aliquots which were distilled in toluene as described by Miller(11). Thus, the difference between means of 7 sets of duplicates by the 2 methods was only 0.13% water. The dried aliquots were mechanically triturated, placed in Soxhlet thimbles within weighing bottles, dried at 100°C for one hour, weighed, extracted for 24 hours with ethyl ether, dried, and reweighed. The weight loss was considered to be total tissue fat since further extraction produced no further weight loss and since known amounts of peanut oil were satisfactorily recovered from de-fatted tissue samples.

Results. Data from 16 normal female albino rats and 14 normal female albino mice were analyzed by the method of multiple regression (12), in which the independent variables were fat-free dry body weight and body fat, and the dependent variable was total body water. In the rats, body water in grams was found to be 25.8 + 2.17 g f.f.d.wt. – 0.05 g body fat. In the mice, body water was 1.52 g + 2.63 g f.f.d.wt. minus 0.12 g body fat. The changes in body water per gram of body fat, –0.05 and –0.12 g respectively, were not statistically significant. Thus, no independent effect of body fat on total body water was demonstrable. Body fat ranged from 24 to 42% of the dry weight in the rats and from 31 to 66% in the mice. Whether excess total water is laid down with fat in one or more of the various forms of experimental obesity is not answered by this study.

Further analysis failed to demonstrate that the intercept values of 25.8 g water in the rats and 1.5 g water in the mice were different from zero. Thus, body water was not shown to be other than a direct proportion of fat-free dry body weight in either group of normal animals.

In Table I the total water is expressed as a percent of fat-free weight in normal and abnormal animals. These data indicate that weanling rats have a slightly higher water fraction in the fat-free body than do grown rats, confirming a previous report(8). There is also a small difference in water fractions between mice and rats. Starvation produces mild decreases, and severe dehydration produces more marked decreases in the water fraction of fat-free tissue. Therefore, these data do not support the generalization that the fat-free body has a "constant" water fraction, although such is very nearly correct for grown, normal mice and rats.

TABLE I. Total Water of Animals Expressed as Percent of Fat-free Weight, 13 Series.

Species, condition, sex	No.	Water, % ± S.D.	Group comparison with series 1, t
Rat; normal, grown ♀	16	72.21 ± .79	
weanling ♀	3	74.73 ± .26	5.41†
grown ♂	7	72.97 ± .73	1.60
no food 120 hr water *ad. lib.*	6	71.65 ± .96	1.40
no food 144 hr water *ad. lib.*	4	71.93 ± .25	.69
no food 168 hr water *ad. lib.*	4	70.88 ± .63	3.12†
no food or water 24 hr	4	71.73 ± .33	2.14*
48	4	70.58 ± .82	3.68†
72	6	69.67 ±1.15	5.95†
84	4	68.35 ±2.85	4.84†
120	3	67.50 ±1.65	8.03†
1% NaCl & food *ad. lib.*, 0.2 cc adren. cort. ext./day	4	71.85 ± .41	.87
Mouse; normal, grown, ♀	14	73.96 ±1.40	4.37†

* $P < .05$. † $P < .01$.

Discussion. The present failure to demonstrate an independent effect of body fat on total body water confirms previous reports in the literature, since multiple regression analyses of the data on guinea pigs(6) and on rats (5) also show no significant independent effect of body fat. The apparent discrepancy mentioned by Keys and Brozek(9) possibly means that some small guinea pigs contained less percent water in the fat-free body, either because the animals were dehydrated or because, as the authors believed(6), water was not completely removed by drying the entire carcass.

Rats that had access to water maintained nearly normal water fractions in the fat-free body for one week, whereas the withholding

of water produced a measureable change in 24 hours (Table I). The lowest fraction that was observed was 64% and this occurred in one rat after 84 hours and in another after 120 hours. None of the rats died or appeared to be moribund prior to sacrifice. The lowest fraction of water compatible with life and possible changes in osmotic pressure of body fluids in severely dehydrated animals remain to be demonstrated. It is probable that similar decreases in the fraction of water in the fat-free body occurred in dehydration studies of 3 and 4 days duration in man(13) and in up to 20 days duration in dogs(14). In both of these studies the concentration of serum NaCl increased.

The attempt to produce chronic edema in rats by means of adrenal cortical extract for 5 days was unsuccessful (Table I). However, others have produced transient retentions of injected water loads equal to 30% of body weight in the rat(15,16). Such a water load would elevate the fraction of water in the fat-free body by about 4%.

If changes in hydration are reflected in changes in total body water expressed as percent of the fat-free body weight in man as they appear to be in the rat, a degree of uncertainty must be considered in the estimation of body fat from total body water in subjects wherein the state of hydration is unknown.

Summary and conclusions. Total body water by vacuum desiccation, total body fat by ether extraction, and fat-free dry body weight were measured in normal mice and rats, and in starved and dehydrated rats. In normal animals total body water was not shown to differ from a direct proportion of fat-free dry weight, and body fat was not shown to have an independent effect on total body water. On the other hand, severe dehydration reduced total body water expressed as percent of fat-free body weight in adult female rats from 72.2 to 67.5. Mice and weanling rats had total body water of 74.0 and 74.7%, respectively, of the fat-free body weight, significantly more than did normal adult rats.

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Isolations of Eastern Equine Encephalomyelitis Virus from Mosquitoes (*Culiseta melanura*) Collected in New Jersey, 1953.* (21411)

PRESTON HOLDEN,† BETTY J. MILLER,‡ AND DANIEL M. JOBBINS.§
(Introduced by W. McD. Hammon.)

From the Department of Epidemiology and Microbiology, Graduate School of Public Health, University of Pittsburgh, Pittsburgh, Pa.

Isolations of Eastern equine encephalomyelitis (EEE) virus from wild caught arthropods have been relatively rare. Howitt, *et al.*(1,2) reported isolations from single pools of chicken mites (*Dermanyssus gallinae* (De Geer) and chicken lice (*Eomenacanthus straminus* (Nitzsch) collected in Tennessee, and from the mosquito *Mansonia perturbans* Walker collected in Georgia. Chamberlain, *et al.*(3) reported an isolation from the mosquito *Culiseta melanura* (Coquillett) collected in Louisiana. This communication describes 2 additional isolations from *C. melanura* and one from an incompletely identified pool of mosquitoes, probably containing *C. melanura,* all collected in New Jersey in 1953.

Materials and methods. 216 arthropod pools were collected on 3 farms in southern New Jersey on which ring-necked pheasants (*Phasianus colchicus torquatus*) were reared. The collection period, August 29 to September 9, coincided with the early stage of an EEE outbreak in each of the 3 pheasant flocks. Mosquitoes were collected principally in light traps operated nightly in the immediate vicinity of the pheasant pens. Mosquitoes for virus isolation were identified under light chloroform anesthesia, hermetically sealed in glass tubes and stored in CO_2 ice chests until tested. In the laboratory, the arthropod pools were triturated in chilled mortars with alundum and 1.5 ml or 3.0 ml of diluent consisting of 33⅓% normal inactivated rabbit serum in phosphate buffered saline (pH 7.4) containing streptomycin and penicillin in final concentrations of 600 to 1,000 μg/ml and 600 to 1,000 units/ml respectively. Following centrifugation at 18,000 rpm for 30 minutes at 5°C, 0.03 ml portions of the supernatant were inoculated intracerebrally into six 3- to 4-week-old mice. On the second or third day, the brains of sick mice were removed aseptically and suspensions were made in 10% rabbit serum-nutrient broth (Difco) for further passage. Viral isolates were identified both by quantitative neutralization tests against known EEE immune serum and by intracerebral challenge of mice immunized against the laboratory strain of EEE virus.‖

Results. In the first series of tests, attempts were made to recover virus from 94 arthropod

TABLE I. Results of Virus Isolation Attempts from 94 Lots of Arthropods Collected in New Jersey, 1953, and Identified under Field Conditions.

Arthropod classification	No. lots	No. individuals	Positive for EEE virus
Aedes sollicitans	11	367	0
Aedes vexans	7	145	0
Anopheles crucians	26	1241	0
Mixed *Culex* species	8	283	1
Culex pipiens	1	50	0
Musca domestica	6	124	0
Phaenicia species	3	39	0
Litter mites (not Dermanyssidae)	32	—*	0
Total	94	2249†	1

* Not counted because of minute size.
† Plus uncounted mites.

* This and related studies on epidemiology of Eastern equine encephalomyelitis in New Jersey provided basis for thesis submitted by senior author to the Graduate School of Public Health of the University of Pittsburgh in partial fulfillment of the requirements for the degree of Doctor of Public Health. The investigation was sponsored jointly by the Communicable Disease Center, Public Health Service, Department of Health, Education and Welfare; New Jersey State Department of Health; Graduate School of Public Health, University of Pittsburgh; and the Commission on Virus and Rickettsial Diseases, Armed Forces Epidemiological Board.

† Communicable Disease Center, Public Health Service, U. S. Department of Health, Education and Welfare, Atlanta, Ga.

‡ Graduate School of Public Health, University of Pittsburgh.

§ Department of Entomology, Rutgers University, New Brunswick, N. J.

TABLE II. Results of Virus Isolation Attempts from 123 Lots* of Arthropods Collected in New Jersey, 1953, and Identified under Laboratory Conditions.

Arthropod classification	No. lots	No. individuals	Positive for EEE virus
Aedes sollicitans	14	606	0
vexans	6	143	0
cantator	4	43	0
cantator & vexans	3	50	0
Anopheles crucians	33	1618	0
Culex pipiens & salinarius†	9	225	0
salinarius	7	98	0
restuans	1	5	0
pipiens†	1	—	0
apicalis	1	2	0
Mansonia perturbans	3	11	0
Culiseta melanura‡	11	131	2
inornata	1	1	0
Stomoxys calcitrans†	8	132	0
Litter mites (not Dermanyssidae)	21	—§	0
Total	123	3065‖	2

* Mosquito lots identified under field conditions as mixed *Culex, A. vexans, A. sollicitans* and *A. crucians* were unsealed and further identified in the laboratory.

† One lot not counted.

‡ 131 individuals found among 13 of 16 tubes labeled in the field as mixed *Culex* species.

§ Not counted because of minute size.

‖ Plus uncounted lots of mites, one lot of uncounted *C. pipiens*, one of mixed *Culex* species and one of *Stomoxys calcitrans*.

pools that had been identified under field conditions (Table I). Of these, a single isolation was obtained from a pool of mosquitoes labeled "mixed *Culex*" which presumably contained only *C. pipiens* and *C. salinarius*. Because neither of these morphologically similar species had been shown to be capable of transmitting EEE virus under laboratory conditions(5), they had not been considered sufficiently important to justify the time required for their separation under field conditions. Following the initial isolation, however, in view of the importance of knowledge as to the specific identity of infected mosquitoes, further testing of arthropods was suspended until the remaining pools were more completely classified in the laboratory.¶ The results of testing 123 lots thus identified are given in Table II. EEE viruses were isolated from 2 of 11 pools of *C. melanura* separated from 13 of the 16 remaining lots of "mixed *Culex*."

The 3 positive lots were all collected in a single trap operated one mile west of Egg Harbor City. One pool was trapped on September 2, the remaining 2 on September 3. The high proportion of "mixed *Culex*" pools known to contain *C. melanura* and the isolation of EEE virus from one mixed pool collected in the same area at the time *C. melanura* was known to be infected strongly suggest that the incompletely identified lot also contained this species.

Each isolate caused sickness or death in all mice from 2 to 4 days following inoculation with original material. In neutralization tests against fifth passage mouse brain stocks, EEE-immune serum protected equally well against the newly isolated and the standard laboratory strains; Western equine encephalomyelitis (WEE) immune serum did not protect (Table III). In vaccination challenge tests, not more than one of nine EEE-immune mice died following inoculation with either a 10^{-2} or 10^{-3} dilution of second mouse passage pools (Table III). Two strains (NJ 78 and NJ 110) produced deaths in greater than 50% of the unvaccinated mice of the same age inoculated with a 10^{-6} or 10^{-7} dilution of the corresponding pools, one strain (NJ 114) in 3 of 4 inoculated with a 10^{-6} dilution.

The presence of virus in the original mosquito suspensions was confirmed by mouse infectivity tests on stored duplicate portions. The LD_{50} titers of these suspensions were 10-3.76, 10-2.80 and 10-2.45, respectively, on the NJ 78, NJ 110 and NJ 114 pools. It is believed unlikely that the isolations could have resulted from laboratory contamination because the use of the stock strain of EEE virus was not permitted in the rooms in which arthropod suspensions were prepared or in which test mice were isolated. In addition, no attempts were made to isolate virus from other field specimens during the time that arthropods were being tested.

Discussion. The isolations of EEE virus

‖ Originally isolated from an equine case by Ten Broeck and Merrill(4) and sent by Dr. Ten Broeck to Miss Beatrice Howitt who subsequently supplied it for Dr. W. McD. Hammon.

¶ Laboratory identifications were made by Dr. Harry Pratt and Mr. Roy Fritz of the Communicable Disease Center.

TABLE III. Results of Neutralization* and Challenge Tests Used to Identify 3 Viral Agents Isolated from Mosquitoes Collected in New Jersey, 1953.

Strain No.	Arthropod identity	Neutralization index		Challenge tests, mortality ratio†/dilution of virus					
		EEE immune serum	WEE immune serum	EEE immune mice	Normal mice				
				10^{-2}	10^{-3}	10^{-6}	10^{-7}	10^{-8}	10^{-9}
NJ 78	"Mixed *Culex*"	264	2	0/5	1/4	—	4/5	2/5	—
NJ 110	*Culiseta melanura*	617	1	0/5	1/4	5/5	5/5	—	—
NJ 114	*Idem*	214	1	0/5	0/5	3/4	1/5	—	—
EEE (lab. strain)	—	151	—	0/4	1/4	4/4	3/4	2/4	1/4

* All tests conducted simultaneously using 4 strains of virus against aliquot portions of immune serums.

† Deaths in numerator; total mice inoculated in denominator.

— Not tested.

from *C. melanura* trapped at the time of epizootics in pheasants strongly suggest that this mosquito is important in the bird-mosquito-bird cycle of infection in New Jersey. This suggestion is supported by 2 virus isolations from naturally-infected English sparrows (*Passer domesticus domesticus*) collected on the same farm on August 25 and September 10(6). While *C. melanura* has not been observed to feed on man it has been observed to feed on birds and has been shown capable of transmitting EEE virus under laboratory conditions(7). In New Jersey the species breeds most abundantly in the late summer and autumn months, the season during which all reported EEE outbreaks in pheasants have occurred.

Summary. Eastern equine encephalomyelitis virus was isolated from each of 2 pools of mosquitoes, *Culiseta melanura* (Coquillett), collected in New Jersey Sept., 1953. A third recovery was made from a pool of mixed mosquitoes quite possibly containing *C. melanura.* Negative results were obtained from similar attempts to isolate EEE virus from more abundant species of arthropods collected concurrently.

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Effect of Cortisone and ACTH on Renal Fat and Limb Regeneration In Adult Salamanders. (21412)

Douglas E. Bragdon* and James Norman Dent.† (Introduced by Jacob Furth.)

From the Departments of Anatomy and Biology, University of Virginia, Charlottesville, Va.

Several groups of investigators have reported that cortisone has an inhibitory effect on healing of skin wounds and bone fractures in several species of mammals, *e.g.*, mice(1), rats(2), rabbits(3,4), and man(5). These and other reports have suggested that interference with the metabolism of tissues of mesenchymal origin is, at least in part, responsible for wound healing retardation. Schotté(6), on the other hand, has reported that injections of ACTH, cortisone, desoxycorticosterone or adrenocortical extract restore the capacity for limb regeneration which has been obliterated in newts by hypophysectomy. The present investigation has been concerned with observations on the influence of cortisone and ACTH on limb regeneration, on adrenal cortical tissue (interrenal tissue) and kidneys of the newt.

Material and methods. Observations were made on a total of 110 adult specimens of *Triturus v. viridescens* (Rafinesque) collected near Charlottesville, Va. The animals of Series I and II (Table I) were collected during the late fall of 1950 and kept inactivated by refrigeration at approximately 4°C until used. Animals of Series III and IV were collected just prior to use. During periods of experimentation, each group of animals was maintained at room temperature in a refrigerator dish (10 x 10 x 20 cm) filled to a depth of approximately 2 cm with tap water which was changed daily. They were fed every second day on ground lean beef fortified with di-basic calcium phosphate and cod liver oil. The right forelimb of each animal was amputated midway between elbow and carpal region on the day following beginning of hormone administration. *Cortisone* was administered both by intraperitoneal injection and by dissolution in water in which animals were kept. Dosages were calculated to correspond on a weight basis to those in mammalian experiments, our animals weighing about 2 to 3 g. In groups A, B, A′, E, and F (Table I) a suspension of cortisone acetate containing 5 mg/ml was diluted to the desired concentration with 0.7% saline, the volume of a single injection being in all instances 0.1 ml. *Control groups* C and C′ received 0.1 ml injections of 0.7% saline. Group D was kept in pond water saturated with cortisone acetate (20 mg/l), the medium changed daily. Groups I and H were kept in tap water containing in solution 40 and 10 mg/l, respectively, of the free alcohol of cortisone. The *ACTH* preparation was Armour Lot #212-74, with a potency 45% of the Armour La-1-A Standard. Dosages of 0.03 mg (Group S) and 0.15 mg (Group T)/animal/day were given in 2 daily injections (morning and evening) in 0.1 ml solution each. *Histological* studies were made of regenerating limbs and of adrenal tissue fixed in Bouin's solution. Segments of kidney with associated masses of interrenal tissue were fixed in a modification of McManus' osmic acid fixative(7).

Results. In no experimentally treated groups was the rate of limb regeneration appreciably slower than that observed in the control groups. In all groups, digit formation began at the end of 4 weeks. There was some variation within groups in the extent to which regeneration had proceeded when animals were killed. On microscopic examination regenerating limbs showed no difference between groups, in thickness or mitotic activity of the epidermis or appearance of dermal tissues.

In animals injected with ACTH a definite darkening of the skin was produced by 0.03 mg and a much more pronounced darkening with 0.15 mg/animal/day. A similar effect has previously been reported by Reinhardt *et al.*(8) and Sulman(9).

In Group A, animals which received 0.5 mg

* Present address: U. S. Public Health Service, Framingham, Mass.

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TABLE I. Procedure Followed in Experiments on Effect of Cortisone and ACTH on Limb Regeneration in the Salamander, *Triturus viridescens viridescens* (Rafinesque).

Series	Group	No. of animals	Treatment	Route of administration	Duration in days	Date terminated
I	A	8	CA*–.5 mg/day	I-P†	12	1/28/51
	B	8	CA—.12 "	"	31	2/16
	C	8	Saline	"	31	"
	D	7	CA—20 mg/l	Sat. sol. in medium		"
II	A′	8	CA—.5 mg/day	I-P†	37	3/26
	E	8	CA—.25 "	"	37	"
	F	8	CA—.17 "	"	37	"
	C′	8	Saline	"	37	"
III	I	9	C-fa*–40 mg/l	In medium	28	5/17
	H	10	C-fa—10 "	*Idem*	28	"
	C″	9	Tap water	"	28	"
IV	R	7	Saline control	I-P†	27	3/14/53
	S	6	ACTH—.03 mg/day	"	27	"
	T	6	" —.15 "	"	27	"

* CA = cortisone acetate; C-fa = cortisone free alcohol.
† Volume of fluid injected = 0.1 ml.

of cortisone acetate per day, one animal died on 4th day, one on 11th, and 2 on 12th day. Because the remaining 4 were obviously in poor condition, they were killed on 12th day. Of a second group (A′) receiving same dosage, only one animal died, 17 days after cortisone was started, suggesting that perhaps some factor other than toxicity of this dose of cortisone was involved. In series I, Group B, one animal died on 19th day. There was one death in each of ACTH-treated groups.

Microscopic examination of adrenal cortical tissue revealed that the cytoplasm was filled with numerous small to medium-sized lipid droplets. These were abundant in both control and treated animals. Except for Group I there was no consistent difference in concentration, abundance, or size variation of droplets in various groups. In Group I the droplets were definitely less abundant suggesting atrophy of the adrenal cortical tissue.

A striking finding in ACTH-treated animals was an increased deposition of fat in the renal tubules. In Group T which received 0.15 mg/animal/day, 5 of the 6 animals showed a definite yellow coloration of the lateral and medial margins of each kidney, the central portion of the kidney not showing the phenomenon. In several animals the yellow coloration was more pronounced posteriorly. Study of sections of osmicated kidney tissue from these animals showed (Fig. 1) that this coloration was caused by a pronounced accumulation of lipoidal material in the cytoplasm of proximal convoluted tubules. There was some fat in lateral tubules of kidneys of the control animals. However, it was distinctly less abundant than in the animals injected with 0.03 mg ACTH/animal/day and these had conspicuously less renal fat than animals which had been maintained on high ACTH dosage regime.

Discussion. The lack of inhibitory effect of cortisone on limb regeneration under the conditions of the experiments here reported may be explained in several ways. It is possible that the doses used were too low and that high dosages would have been inhibitory. Significantly, Ragan(10) stated that to induce depression of fibroplasia in experimental animals he and his associates gave "as much cortisone as we can get away with and still keep the animal alive." He also(10) reported species differences among mammals with respect to the retardation of wound healing by cortisone. Findley and Howes(11) found that the effect of cortisone on wound healing is influenced by nutritional status of the animal, the inhibitory effect being greatest in the presence of protein depletion. Since our animals were fed on a diet of ground lean beef, it is perhaps not surprising that no inhibitory effect was noted at the cortisone dosages used.

The adrenal glands of frogs injected with

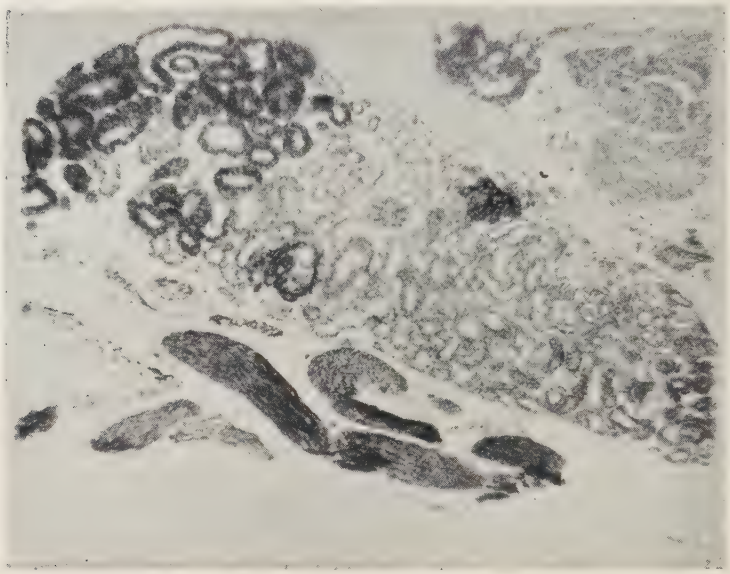

FIG. 1. Low power view of kidney section containing interrenal mass. Marked lipid accumulations visible both in interrenal mass at right of photograph, in walls of lateral renal tubules at left and in myelinated nerves at bottom (×45).

ACTH showed some hyperplasia but no hypertrophy(12). Miller(13), studying *Triturus torosus,* suggested for the first time the possibility that the response of interrenal cells in the urodele to ACTH varies with gonadal maturation cycle. Our findings are consistent with those of Miller. The gonads of our animals were all in the breeding phase. Like those of Miller, their adrenal cortical tissue did not show consistent changes following administration of cortisone or ACTH except among animals of Group I. Since the ACTH potency of our preparation was only about 50% that of the standard La-1-A, our higher dosage of 0.15 mg/animal/day is roughly only 2½ times the dose which he found to be ineffective during the breeding season and is only 1/6–1/7 as potent as the dose which he found to produce degenerative changes. It is of interest that cortisone alcohol dissolved in water in which animals of Group I were living was apparently absorbed in sufficient quantity to produce cortical atrophy, presumably by suppression of pituitary adrenocorticotrophic hormone, yet it did not inhibit the regeneration of their severed limbs.

Effects of ACTH on fat metabolism have been indicated in several communications (14-17) although the various findings are somewhat contradictory. The renal deposition of fat described here has not previously been reported. Further work will be required to establish its significance.

Summary. 1. To 10 groups of 6 to 10 adult specimens of *Triturus v. viridescens* cortisone acetate, cortisone alcohol, or ACTH was administered for periods of 27-37 days. Four groups of control animals were injected with saline or maintained in tap water or saline. The right forelimbs of all animals were amputated midway between the elbow and carpal region one day following the beginning of treatment. 2. There was no appreciable retardation of limb regeneration in any of the groups. 3. Marked deposition of fat was observed in the lateral renal tubules of animals treated with ACTH, the concentration deposited being approximately proportional to the dose of ACTH. 4. Adrenal cortical tissue of salamanders maintained in the higher concentration of cortisone alcohol (40 mg/l) was found to have undergone a reduction in the lipid droplets which are characteristically present in the normal gland.

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Inactivation of Oxytocin by Homogenates of Uteri and Other Tissues from Normal and Pregnant Rats. (21413)

WILBUR H. SAWYER.*

From the Biological Laboratories, Harvard University, Cambridge, Mass.

The successful maintenance of pregnancy and the avoidance of premature delivery requires that the uterine muscle remains relatively quiescent throughout gestation. Not until term may the powerful contractions of labor intervene. The strong spontaneous motility characteristic of the myometrium must be suppressed, and, since circulating neurohypophyseal hormone must be present during pregnancy, the response to this potent oxytocic agent must also be controlled. In the rat progesterone treatment or pregnancy do not significantly reduce uterine motility(1) or sensitivity to the neurohypophyseal hormone (2,3) as measured *in vitro*. If failure to induce abortion can be used as an index of *in vivo* responsiveness the uterus of the pregnant rat is very resistant to the effects of injected oxytocin(4,5). This rather paradoxical indifference of the pregnant rat myometrium *in situ* has led us to look about for possible explanations.

It has been repeatedly demonstrated that blood, serum, or plasma from pregnant women contains a high concentration of an enzyme capable of inactivating oxytocin(6,7). This "oxytocinase" has not been found, however, in plasma from pregnant rats, rabbits, or guinea pigs(8). The present paper reports the examination of some of the tissues of the rat to determine if they are capable of destroying neurohypophyseal oxytocic activity *in vitro* and if changes in this ability occur during pregnancy and pseudopregnancy.

Methods. The rats used were of the Harvard strain. Vaginal smears were taken daily and the appearance of sperm signalled the first day of pregnancy. The pseudopregnant rat tissues were supplied by Dr. Joseph T. Velardo and had been prepared by methods previously described(9). Blood was collected in a heparinized syringe from the heart under ether anesthesia. Tissues were removed immediately and homogenized in 10 volumes of 0.1 N phosphate buffer at pH 6.5, or were quickly frozen for later use. Each tissue homogenate was serially diluted with cold buffer so that each tube contained 1 ml of homogenate di-

* Present address: Department of Physiology, New York University College of Medicine, New York City.

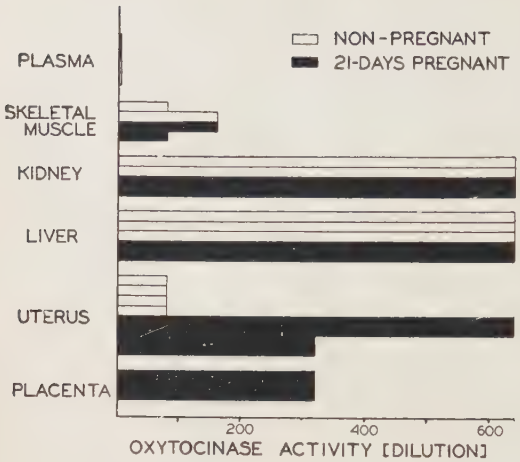

FIG. 1. Comparison of oxytocinase activity in pregnant and non-pregnant rat tissues.

luted 1/10, 1/20, 1/40, etc. Pituitrin in buffer, 1 ml containing 100 milliunits, was added to each tube. One control tube containing homogenate only, and another containing pituitrin only, were included in each run. The tubes were incubated in a water bath at 37°C for exactly one hour and then placed in boiling water for 5 minutes to stop digestion. The *oxytocic activity* of the contents of each tube was then compared to that of the tube containing pituitrin alone. The enzymatic activity of the homogenate is expressed in terms of the highest dilution at which at least 50% of the added pituitrin was destroyed during the period of incubation. Oxytocic activity was determined by a modification of the Holton (10) rat uterus assay.

Results. Heparinized rat plasma diluted ¼ does inactivate pituitrin to a slight extent after one hour at 37°. Pregnancy plasma is not appreciably more active in this respect than is normal plasma. Kidney and liver homogenates are highly active but there is no detectable increase in activity during pregnancy. Non-pregnant uteri, and skeletal muscle from pregnant and non-pregnant rats, show a considerable degree of oxytocinase activity and are all in a comparable range. Uteri from pregnant rats, however, show a 4-to-8-fold increase in the ability to destroy oxytocin, and are, on a weight basis, often as potent as liver or kidney in this respect (Fig. 1).

If the oxytocinase activity of uterine muscle is compared to that of the placenta it is found that at both 14 and 21 days of pregnancy there is more activity in the muscle, although placental homogenates do show a considerable concentration of the enzyme (Fig. 1).

If the oxytocinase activity of uteri taken from rats at several stages of pregnancy are compared (Fig. 2) it is seen that there is a suggestion of an increase at 7 days, and, by 14 days, this has become quite marked. The enzyme activity of the uterus at delivery, or shortly post partum, however, has returned to the 7 day level.

Uteri from pseudopregnant rats show little or no increase in oxytocinase while homogenates of uteri containing massive deciduomata have approximately 4 times the activity of normal non-pregnant uteri.

Discussion. It appears quite certain that the neurohypophyseal hormone is not essential for normal parturition in the rat. If it has any physiological function as an oxytocic agent it must be sheerly complementary to other factors that govern labor. The fact that this hormone has a particularly violent effect on the myometrium and the probability that it is present during pregnancy should necessitate rather extraordinary measures designed to defend the uterine contents from premature delivery.

The tremendous increase in circulating oxytocinase in human pregnancy may represent such a defensive mechanism. The increase in this enzyme in the rat uterine muscle during pregnancy is, quantitatively, less impressive.

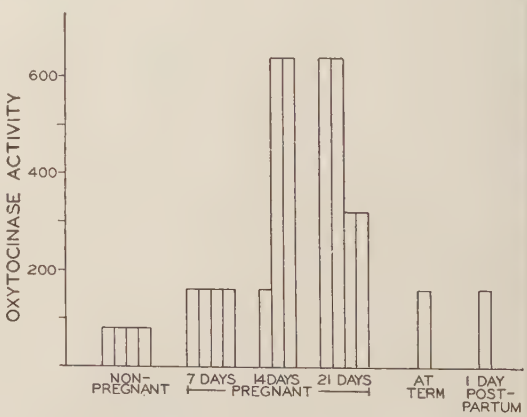

FIG. 2. Oxytocinase activity of myometrium at different stages of pregnancy in the rat.

The fact that there is a sharp decrease in oxytocinase activity at the time of delivery, however, indicates that its presence is not an incidental result of myometrial hypertrophy, and that it may play some role in the preservation of uterine quiescence until term. The presence of the enzyme in the end organ itself, the uterine muscle, in concentrations similar to that in liver or kidney, lends further weight to this hypothesis.

The experiments reported here are exploratory in nature. It would appear that this is a promising line for future investigation that may help shed more light on the mysteries surrounding the changing physiological conditions of the myometrium at term that make normal parturition possible.

Summary. Homogenates of rat tissues have been tested for their ability to inactivate neurohypophyseal oxytocin. There is a striking increase in oxytocinase activity in uterine muscle during pregnancy.

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Effect of Magnesium Chloride and pH on Heat Inactivation of Fatty Acid Oxidase of Mitochondria.* (21414)

ROBERT F. WITTER, ETHEL H. NEWCOMB, MARY A. COTTONE, AND ELMER STOTZ.
(Introduced by Alexander L. Dounce.)

From Department of Biochemistry, University of Rochester School of Medicine and Dentistry, Rochester, N. Y.

The marked instability of the oxidative systems of mitochondria is a serious handicap in the assay of these enzymes. Previous work has shown that some factor or factors present in the reaction mixture commonly used to study the fatty acid oxidase of liver mitochondria protects the system against inactivation by heat(1-4). Therefore, is seemed desirable to investigate further the nature of these unknown protective factors.

The experiments reported in this paper were designed to reveal which components of the reaction mixture might be responsible for the protective effect. Sorbic acid was used as a model substrate with rat liver mitochondria as the source of the fatty acid oxidase. In these studies, the enzyme preparation was preincubated with various components of the reaction mixture, and the subsequent ability of the system to oxidize sorbic acid was then determined. The oxidation of sorbate was known from previous work(5) to be absolutely dependent on the cooxidation of an activator, in this case either α-ketoglutarate or malate. A loss in ability to oxidize either of these dicarboxylic acids would lead to an apparent decrease in sorbic acid oxidase activity. Accordingly, studies of the effects of preincubation of the mitochondria on their subsequent ability to oxidize either α-ketoglutarate or malate were also carried out. In addition, since fatty acid oxidation is known to be inhibited by physical damage to the mitochondria(1-4,6), a study was made of the destructive action on the mitochondria of preincuba-

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tion with various components of the reaction mixture. Some of the data in this study were given before the Fed. of Am. Soc. for Exp. Biol(7).

Methods. Studies of the effect of preincubation of the mitochondria on their sorbic acid oxidase activity were conducted in the following manner. The mitochondria and those components of the reaction mixture whose protective effect was to be tested were placed in the main compartment of a chilled Warburg flask; the remainder of the reaction mixture was placed in the side arms. After a preincubation period of 5 minutes at 30°, the contents of the side arms were dumped, thus initiating the oxidation of sorbate. This was allowed to proceed for 30 minutes at 30°. As a control with each experiment, the sorbic acid oxidase activity of previously unincubated mitochondria was also measured. For these controls the preincubation period was omitted, and the mitochondria were added to the complete reaction mixture at 0° and transferred to the bath at 30° where the oxidation was allowed to proceed for 30 minutes. Studies of the effect of preincubation of the mitochondria on their subsequent ability to oxidize α-ketoglutarate or malate were conducted in a similar manner except that sorbate was omitted from the reaction mixture. The *complete reaction mixture* contained the following constituents: 0.02 M tris(hydroxymethyl)amino methane ("tris") buffer, pH 8.0; 6.6 x 10^{-3} M potassium phosphate, pH 8.0, 6.6 x 10^{-3} M magnesium chloride, 3.3 x 10^{-4} M α-ketoglutarate or malate, 3 x 10^{-6} M cytochrome *c*, 9 x 10^{-4} M ATP, 1 x 10^{-3} M sorbate, and 0.04 to 0.05 M potassium chloride (this includes the salt in 0.2 ml of enzyme suspension) in a total volume of 3.0 ml. Since the volume of the *preincubation mixture* varied from 2.2 to 2.8 ml, the concentration of certain constituents in the latter may have been higher than that of the complete reaction mixture in some experiments by a maximum of 30%. However, the final osmolar concentration of salts in both the preincubation mixture and the final reaction mixture was maintained at a value equivalent to 0.08 M or 0.09 M monovalent salt by appropriate distribution of the potassium chloride between the side arms and the main compartment. The *phosphate buffer* was freed of heavy metals by hydrogen sulfide. The sources and purity of the other chemicals have been described in previous publications(5,7-10). Sorbic acid was determined in distillates of metaphosphate filtrates as previously described(8). Oxygen uptake was measured in the Warburg apparatus. Mitochondria from rat liver were prepared by hypertonic sucrose isotonic potassium chloride procedure of Kennedy and Lehninger(11). These preparations were washed 3 times with isotonic potassium chloride and finally suspended in the same medium. The *rate of change* in turbidity of mitochondrial suspensions was used as index of mitochondrial damage. This was measured in a Beckman spectrophotometer by the change in optical density at 520 mμ of suspensions of mitochondria over a 5 minute period at 30° as described by Cleland(12). Except for the mitochondria, each constituent was at the same concentration as in the reaction mixture, and the osmolar concentration of salts was maintained equivalent to 0.09 M potassium chloride by suitable adjustment of the concentration of this salt.

TABLE I. Effects of Preincubation on Subsequent Ability of Mitochondria to Oxidize Sorbate. Each sample except the control was treated with a preincubation mixture as shown in the table for 5 min. at 30° before the oxidation of sorbate was initiated by dumping in the rest of the reaction mixture. In this experiment the activator was malate but almost identical results were obtained when α-ketoglutarate was used.

Preincubation mixture		Relative sorbate oxidase activity, %
Control, complete reaction mixture, pH 8.0		100
Reaction mixture complete except for sorbate		103*
KCl, pH 6.7		27
KCl, tris,† pH 8.0		61
Idem	$MgCl_2$	93
"	$MgCl_2$, ATP	100
"	ATP	67
"	adenylic acid	60
"	PO_4	6
"	PO_4, $MgCl_2$	90
"	PO_4, ATP	51

* The extra 3% is due to experimental error.
† "tris" or tris(hydroxymethyl)aminomethane buffer.

Results. In Table I are illustrated the effects

TABLE II. Effects of Preincubation on Subsequent Ability of Mitochondria to Oxidize Dicarboxylic Acids. 2 experiments. The experiments were the same as in Table I except that sorbate was omitted from the reaction mixture. Each sample except the unincubated control was treated with a preincubation mixture as shown in the table for 5 min. at 30° before the oxidation of the dicarboxylic acid was initiated by dumping in the rest of the reaction mixture.

Exp. No.	Preincubation mixture	Dicarboxylic acids as μm of oxygen/hr	
		α-Keto-glutarate	Malate
1.	Control "complete reaction mixture," pH 8.0	3.15	2.00
	KCl, pH 6.7	1.80	.95
	KCl, tris,* pH 8.0	3.05	1.90
2.	Control "complete reaction mixture," pH 8.0	2.50	1.70
	KCl, tris, pH 8.0	2.20	1.90
	Idem $MgCl_2$	2.40	1.90
	" PO_4	.56	.32
	" PO_4, $MgCl_2$	2.30	2.20

* "tris" or tris(hydroxymethyl)aminomethane buffer.

of preincubation of the mitochondria under various conditions at 30° on their subsequent ability to oxidize sorbic acid. Other factors were also varied but without effect, and have therefore been omitted from the table. The amount of the acid which was oxidized is expressed as per cent of the amount oxidized in the control sample which was not preincubated. In Table II are shown similar experiments with α-ketoglutarate or malate. Here, the results are given as oxygen uptake. The mitochondria used in this study were suspended in potassium chloride solutions after isolation. Such suspensions were found to have a pH of 6.5 to 6.7 which did not change on incubation at 30°, whereas the complete reaction mixture is buffered at pH 8.0 with "tris" and phosphate buffers.

From the data presented in Tables I and II the following conclusions can be drawn. The alkaline pH of the reaction mixture and the presence of magnesium chloride were the two most important factors in the prevention of heat inactivation of the sorbic acid oxidase. The phosphate buffer was the most destructive factor. The alkaline pH of the reaction mixture, brought about by the "tris" buffer, prevented a loss in the ability of the system to oxidize α-ketoglutarate or malate, the necessary activators of the oxidation of sorbate, and thus apparently protected the fatty acid oxidase in an indirect manner. However, this provided only partial protection for the sorbic acid oxidase, since some heat activation was still observed. The addition of magnesium chloride to the alkaline suspension before preincubation completely protected the sorbic acid oxidase against heat inactivation but had no additional effect on the oxidation of α-ketoglutarate or malate. When the mitochondria were preincubated with phosphate instead of tris buffer, pH 8.0, the oxidation of α-ketoglutarate, malate, and sorbate was almost completely blocked. These effects of phosphate were completely prevented by simultaneous preincubation with magnesium chloride.

These results indicate that magnesium performs two important roles in the protection of the sorbate oxidase from heat destruction. First of all this cation directly protects the oxidase system. Secondly magnesium chloride counteracts the deleterious effect of phosphate on the oxidation of the activators of sorbate oxidation.

Fatty acid oxidation is very sensitive to procedures which damage mitochondria(1-4, 6). The studies of Cleland(12) and Raaflaub (13) indicate magnesium inhibits(13) whereas phosphate accelerates(12,13) the swelling of mitochondria exposed to hypotonic conditions. In order to investigate the possibility that these observations might explain the opposite effects of magnesium chloride or phosphate on sorbic acid oxidation, the effects of these substances on the stability of mitochondria was investigated by a turbidimetric procedure as described in the experimental section. Although the turbidity was propor-

TABLE III. Change in Optical Density of Suspensions of Mitochondria in Hypotonic Potassium Chloride in Presence of ATP, $MgCl_2$ or Phosphate. Initial optical density was 0.330. Other details are in the experimental section.

Additions		△-density, 5 min. at 30°
KCl, pH 6.5		—.100
KCl, tris pH 8.0		—.120
Idem	PO_4 pH 8.0	—.220
Water, tris pH 8.0		—.240
KCl, tris pH 8.0, $MgCl_2$		+.010
Idem	ATP	+.030
"	adenylic acid	—.200
"	PO_4, $MgCl_2$	—.005
"	PO_4, ATP	+.010

tional to the concentration of mitochondrial suspension added, it is obvious that this method can reveal only gross damage to the mitochondria. However, this procedure appeared to be satisfactory for these studies. The results of these experiments are given in Table III.

First of all in dilute potassium chloride buffered at pH 8 with tris buffer there was a gradual decline in optical density of the suspension. This was markedly accelerated by phosphate, in fact the change was the same as that induced by suspension of the mitochondria in water. These effects of suspension in phosphate or potassium chloride could be prevented by the presence of magnesium chloride or ATP but not by adenylic acid. An osmolar equivalent amount of potassium chloride did not have the same effect as magnesium chloride, hence these effects are specific for the latter salt. The facts that both ATP and magnesium chloride protected the mitochondria from damage (Table III) whereas only magnesium chloride protected the sorbic acid oxidase (Table I) indicates that this salt had some more specific effect than merely the stabilization of the mitochondria to hypotonic conditions. The same thing appears to be true for the protective action of solutions of higher pH on the oxidation of α-ketoglutarate or malate, since reference to Table III will show that the decrease in turbidity was the same in suspensions at pH 8.0 as at pH 6.5, whereas only solutions with the former pH had a protective effect on the oxidation of these dicarboxylic acids.

On the other hand the inhibitory action of phosphate appears to be due to the damaging effect of this anion on the mitochondria. This is shown by the facts that preincubation with phosphate causes great damage to the mitochondria and almost complete inhibition of sorbic acid oxidase activity, whereas when ATP is also present in the preincubation mixture the damage to the mitochondria is prevented (Table III) and phosphate is no more inhibitory to the sorbate oxidase system than is chloride ion.

The actual mode of action of magnesium chloride is not revealed by these experiments. However, in view of the requirements of fatty acid oxidation for oxidative phosphorylation (3,4,8,11), and the sensitiveness of the latter process to heat inactivation(14-18) it is suggested as a working hypothesis that magnesium chloride may act to protect those enzymes responsible for oxidative phosphorylation.

Summary. In the reaction mixture alkaline pH 8 and magnesium chloride were the most important factors in prevention of heat inactivation of the sorbate, α-ketoglutarate, or malate oxidase of rat liver mitochondria. The most destructive factor was the presence of phosphate. Either magnesium chloride or ATP prevented, and phosphate accelerated, the destruction of mitochondria in hypotonic solution, but magnesium chloride had a specific protective effect on sorbic acid oxidase in addition to its effect of preventing destruction of the mitochondria.

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Renin Inhibition of Compensatory Renal Hypertrophy.* (21415)

C. A. SCHAFFENBURG,† G. M. C. MASSON, AND A. C. CORCORAN.
(With the technical assistance of Devina C. Tweed.)
From the Research Division, Cleveland Clinic Foundation, and The Frank E. Bunts Educational Institute, Cleveland, O.

High protein diets, androgens, somatotrophin and thyroxine, all of which elicit renal hypertrophy and enhance "compensatory" hypertrophy of the kidney, have also a pronounced effect on protein turnover. These observations led Braun-Menéndez(1) to postulate a renotrophin, possibly a protein metabolite, capable of stimulating kidney hypertrophy and active in the genesis of renal and hormonal hypertension.

The kidney, because of its secretion of renin into the blood, can be considered an endocrine organ and therefore might be subject to a self-regulating trophic mechanism such as acts in the case of other endocrine glands. In the case of the kidney, compensatory hypertrophy after uninephrectomy might be regulated by circulating renin or a similar renal secretion. This concept was tested in uninephrectomized otherwise normal rats and in hypophysectomized animals.

Methods. Female albino rats were used in all experiments. Intact animals weighed an average of 150 g. Hypophysectomy was performed at a body weight of about 60 g; these animals were kept for a month until they attained a constant body weight. Completeness of hypophysectomy was verified at autopsy by inspection of the sella turcica and by atrophy of adrenals and ovaries. All animals were housed in a constant temperature room and received a diet of Purina dog chow with fresh water *ad lib.* Hypophysectomized animals were given in addition a supplement of horse meat, fresh oranges and bread. For test, left nephrectomy was done under ether anesthesia; the kidney was blotted free of blood and weighed fresh on a torsion balance. At the end of the experimental period the rats were sacrificed with chloroform, the right kidney was removed and weighed similarly. Kidney solid and water contents were determined in some experiments. For these, kidneys were deposited into tared weighing bottles containing dried preservative(2). They were then minced with scissors and placed in an oven to constant weight. Right kidney hypertrophy was estimated in 3 ways: a) from the absolute (right kidney minus left kidney) or relative $\left(100\,\frac{\text{right kidney minus left kidney}}{\text{left kidney}}\right)$ differences in total weights; b) from the differences in their solid content; c) in experiments testing the inhibition of hypertrophy, total mean relative weight gain was considered as 100% in untreated uninephrectomized controls and the relative kidney weight gains in experimental groups were expressed in terms of this

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† Present address: Research Department, Mount Sinai Hospital, Cleveland, O.

TABLE I. Effect of Renin on Compensatory Renal Hypertrophy and Effect of Time Thereon.*

Group	No. rats	Treatment	Duration in days	Right kidney hypertrophy: Total (mg)	Right kidney hypertrophy: Solids (mg)	% increase over left kidney: Total	% increase over left kidney: Solids	Body wt change, g
I A	3	None	1	182.3 (102.0/329.0)	32.8 (18.8/58.9)	27.2 (15.1/46.8)	21.1 (12.5/35.4)	− 7.3 (−6/−8)
B	3	Renin, 56 U	1	71.8 (23.2/123.4)	15.3 (3.5/35.0)	12.6 (4.2/22.0)	11.6 (2.4/26.7)	− 1.6 (−14/+6)
II A	3	None	2	112.3 (85.0/147.8)	27.9 (18.3/34.7)	18.9 (12.6/27.4)	20.0 (11.3/27.4)	− 3.3 (−2/−5)
B	3	Renin, 112 U	2	33.0 (−4.6/90.5)	7.6 (1.9/17.2)	5.3 (−.67/14.8)	5.2 (1.24/11.8)	−20 (−16/−24)
III	6	None	4	147.4 (118.0/169.4)	35.6 (32.6/39.1)	26.3 (20.6/33.8)	27.2 (24.3/32.5)	− 1 (−3/+3)
IV	5	"	8	221.2 (160.2/299.2)	52.2 (40.9/71.0)	34.7 (28.2/42.8)	37.4 (32.1/45.2)	+ 1 (−17/+22)
V	6	"	16	322.4 (210.4/490.4)	69.3 (50.9/98.3)	56.1 (37.6/94.0)	51.0 (38.4/78.0)	+26.3 (−28/+49)
VI	6	"	32	465.2 (365.8/564.4)	93.3 (80.3/104.9)	78.0 (64.0/87.2)	65.8 (57.5/72.4)	+39.5 (+28/+49)

* Figures in parentheses represent spread.

standard. Two preparations of renin‡ were available: one, used in the first experiment, was a renal extract (Dr. A. Green preparation #64, to be called hereinafter renin I) containing 15 mg of protein per ml with a pressor activity of 1.9 Goldblatt dog units per mg protein. The second, used in subsequent experiments, was a lyophilized powder (Dr. E. Haas preparation #38, hereinafter referred to as renin II) originally dissolved in a 0.01M pyrophosphate buffer and equivalent to 5.4 Goldblatt dog units per mg protein. This powder was dissolved in distilled water in concentrations suitable for injection. For experiments with inactivated renin the preparation was treated as follows: solution of renin II was brought to pH 11-12 at room temperature for 15 minutes with concentrated NaOH, and then brought down to pH 7 with 0.1 N HCl(3). Renin was administered according to two schedules: schedule A, consisted in 4 injections of 0.5 cc each, beginning immediately after left nephrectomy (9 a. m., 1, 5 and 9 p. m.); schedule B involved two additional injections given at 1 and 5 a. m.

Results. Exp. 1. This was an orienting experiment concerned with the time course of compensatory hypertrophy and the possible effect thereon of a brief period of renin administration. Six groups of 6 animals each were observed. These were sacrificed at different times after left uninephrectomy (Table I) for determination of right kidney weight, solid and water contents. Half of the rats in group I (24 hr) and half in group II (48 hr) were given renin I subcutaneously according to schedule A. As shown in Table I, right hypertrophy was already present at 24 hr after left uninephrectomy. Sudden weight gain involved a true increase in renal substance since it was associated with a 20% increase in solid content. Hypertrophy increased with time to reach about 70-80% of original kidney mass at 32 days. The data indicate that fresh kidney weight gain at 24 or 48 hours may, under standard conditions, be used as presumptive evidence of compensatory hypertrophy. Uninephrectomy caused loss of body weight from which the animals began to recover at about 8 days. Renin treatment inhibited compensatory hypertrophy; this effect was greater in animals treated for 48 hours. All animals, except two, lost weight in the 24-hour renin-treated group.

Exp. 2. To determine the minimal dose of renin effective in inhibiting hypertrophy as well as the desirable route of administration, experiments were carried out using renin II, schedule B, over 24 hours, subcutaneously and

‡ We are indebted to Dr. A. Green formerly of this Division, and to Drs. H. Goldblatt and E. Haas, Mount Sinai Hospital, Cleveland, for the renin used.

TABLE II. Effect of Subcutaneous and Intraperitoneal Renin on Compensatory Renal Hypertrophy.*

Group	No. rats	Treatment	Right kidney hypertrophy: Total (mg)	% over left kidney	% of controls
I	2	Renin, 60 U	29.5 (14.1/45.0)	4.8 (2.3/7.3)	20.0
II	2	20 U	99.1 (79.4/118.8)	16.4 (12.3/20.6)	68.0
III	2	5 U	57.7 (44.2/71.2)	10.1 (7.8/12.4)	39.8
IV	2	1 U	121.0 (117.6/124.4)	18.8 (18.3/19.4)	82.9
V	2	60 U		Rats died	
VI	2	20 U	44.3 (30.4/58.2)	6.59 (4.5/8.7)	30.5
VII	2	5 U	53.0 (48.0/58.0)	7.36 (6.7/8.1)	36.5
VIII	2	1 U	74.3 (73.6/75.6)	11.80 (11.1/12.5)	51.3
IX	8	0 (controls)	144.7 (78.2/255.2)	23.6 (10.5/46.2)	100.0

* Renin administered subcutaneously to animals of groups I to IV, and intraperitoneally to groups V to VIII.

intraperitoneally. Data from fresh kidney weights appear in Table II.

Renin II was distinctly more active when given intraperitoneally than subcutaneously. Intraperitoneally, the highest dose (60 U.) was lethal. The renal response was roughly proportional to the dose with intraperitoneal administration. Proportionality was not apparent at the 20 U. level in the subcutaneous series. The means indicate responses to 5 U. in both series; these responses were small, so that a dose of 20 U. or more given intraperitoneally was used in subsequent experiments.

Exp. 3. Loss of body weight after uninephrectomy was intensified by administration of renin over 48 hours (Table I). This expresses a catabolic effect of renin(4) which might of itself hamper renal hypertrophy. Accordingly a group of 33 uninephrectomized rats was divided into 3 groups. Animals of groups I and II were treated with cortisone acetate or 2, 4-dinitrophenol during 24 and 48 hours. The total daily dose (2.5 mg for each compound) was given in 4 subcutaneous in-

TABLE III. Effect of 2, 4-Dinitrophenol (DNP) and Cortisone Acetate on Compensatory Renal Hypertrophy.

Group	No. rats	Treatment	Duration in hr	Right kidney hypertrophy: Total (mg)	Solids (mg)	% increase over left kidney: Total	Solids	Body wt change, g
I A	5	DNP, 2.5 mg	24	114.8 (90.6/147.2)	22.1 (13.1/34.3)	18.3 (11.5/22.7)	14.9 (9.8/23.2)	− 4.4 (0/−9)
B	6	DNP, 5.0 mg	48	131.4 (71.8/194.8)	21.3 (10.5/36.4)	22.0 (12.8/34.4)	14.8 (7.7/27.0)	−20 (−17/−24)
II A	6	Cort., 2.5 mg	24	88.1 (38.2/117.0)	16.5 (11.2/22.3)	13.6 (7.6/17.0)	11.0 (8.4/13.6)	− 6.5 (−3/−11)
B	6	Cort., 5.0 mg	48	116.7 (84.6/136.6)	22.5 (15.1/28.3)	19.1 (15.2/21.7)	15.4 (10.5/19.6)	−15.1 (−12/−17)
III A	6	None	24	102.2 (75.6/159.0)	19.5 (15.3/23.7)	16.5 (11.9/28.5)	13.3 (11.2/17.7)	− 4.7 (−1/−9)
B	4	"	48	103.5 (73.8/145.6)	19.6 (13.8/25.7)	18.3 (13.3/26.4)	14.8 (9.1/19.2)	−10.2 (−5/−15)

TABLE IV. Effect of Time on Compensatory Renal Hypertrophy in the Hypophysectomized Rat.

Group	No. rats	Duration in hr	Right kidney hypertrophy				Body wt change, g
			Total (mg)	Solids (mg)	% increase over left kidney Total	Solids	
I	7	24	39.0 (18.2/66.0)	2.8 (−1.2/+10.7)	17.2 (8.9/27.0)	5.0 (−2.2/20.4)	− .1 (−4/+2)
II	7	48	41.3 (22.0/64.4)	3.3 (−1.5/8.1)	17.7 (8.4/32.0)	5.8 (−2.3/13.9)	−2.7 (−1/−5)
III	6	72	55.5 (44.8/62.0)	8.0 (4.7/10.9)	24.1 (19.8/28.2)	14.3 (8.5/20.0)	− .3 (−3/+2)
IV	6	96	84.2 (59.2/110.0)	8.3 (−2.6/14.1)	37.0 (28.0/42.8)	14.0 (−5.4/21.7)	+ .8 (−1/+3)

jections. Animals of Group III were untreated uninephrectomized controls. Autopsy was performed at 24 and 48 hours, and wet and dry kidney weights were determined. The data in Table III show that body weight loss was evident in the two 48-hour groups treated with cortisone or dinitrophenol; however neither agent caused an inhibition of right kidney hypertrophy.

Exp. 4 and 5. Two experiments were carried out in hypophysectomized animals. In experiment 4 the degree of right kidney hypertrophy was determined at different time intervals following unilateral nephrectomy. Results are recorded in Table IV. Although there was an increase in right kidney wet weight 24 hours after uninephrectomy, this did not involve a proportionate increase in solid content. There was no appreciable change in body weight. In Exp. 5, hypophysectomized uninephrectomized rats received intraperitoneal injections of renin II according to schedule B over 24 hours. The results (Table V) showed that renin did not prevent gain in kidney weight and, as compared to untreated controls, did not alter body weight.

Exp. 6. Six uninephrectomized rats treated for 24 hours with inactivated renin II, given intraperitoneally according to schedule B, in a dose equivalent to "30 U.," showed no response compared to untreated, uninephrectomized controls (100% hypertrophy for controls and 96.7% for treated animals).

Discussion. These experiments show that renin inhibits compensatory renal hypertrophy. Assuming that it could exert this effect over long, as well as short periods, and that, as seems likely, it is a renal hormone, then a self-regulatory "servo" function in the control of kidney growth can be added to the other activities (pressor, diuretic, natriuretic, proteinuretic, corticotrophic, catabolic) (4-8) of renin. Braun-Menéndez(1) had postulated that a hypothetical protein metabolite, or metabolites, might both stimulate renal growth and provoke hypertension. In the light of the present experiments, the pressor aspect of the action of "renotrophin" would be exerted by the known pressor agent renin. Release of renin might be elicited as a response to any renotrophic stimulus. This concept would account for the varying increases of arterial pressure found in several circumstances (unilateral nephrectomy, high protein diets, administration of thyroxine, 17-methylandrostenediol or somatotrophin) (1,9-11) associated with renal growth.

The mechanism of this action of renin is not clear. It is not a nonspecific response to injected inactive protein. The severe catabolic state elicited by prolonged cortisone overdosage does interfere with compensatory renal hypertrophy(12); however such interference is not demonstrable in the catabolic states induced by brief periods of treatment with either dinitrophenol or cortisone. The inhibitory effect of renin is therefore probably unrelated to its catabolic effect.

The observation that renin fails to exert an inhibitory effect in hypophysectomized animals bears on other aspects of the action of renin which are conditioned by pituitary-adrenal cortical function(5-7, 13). From the present experiments the mechanism of action cannot be established. The loss of body weight which occurs after uninephrectomy in

TABLE V. Effect of Intraperitoneal Renin on Compensatory Renal Hypertrophy in the Hypophysectomized Rat.

Group	No. rats	Treatment	Right kidney hypertrophy: Total (mg)	% over left kidney	% of controls	Body wt change, g
I	5	Renin, 20 U	65.6 (31.6/86.4)	26.7 (13.3/36.4)	121	−2.8 (−2/−4)
II	5	5 U	48.3 (36.0/65.0)	21.1 (14.9/30.9)	96.4	−1.8 (−1/−3)
III	8	None	49.0 (33.6/71.2)	22.0 (15.2/33.2)	100	−1.1 (0/−2)

treated and untreated animals, and its failure to appear after hypophysectomy, deserves further study.

Summary. Renin inhibits compensatory renal hypertrophy and is more active intraperitoneally than subcutaneously. This inhibition, which is caused by renin as such, is not related to the catabolic effect of renin; it is abolished by hypophysectomy. Our experiments suggest that renin, like other hormonal secretions, can act in a self-regulating mechanism in the control of organ growth.

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Rapid Development of Portal Fatty Liver in Rats Consuming Various Plant Materials.*† (21416)

MAURICE E. SHILS, IRA FRIEDLAND, AND W. B. STEWART.

From the School of Public Health and Department of Pathology, College of Physicians and Surgeons, Columbia University.

In a previous study(1) we showed that rats maintained on a diet high in corn but low in animal protein developed accumulations of visible fat in portal portion of the lobule; this condition occurred with great regularity in second generation rats born of mothers maintained on the same diet. It is our purpose to show that this disturbance can also be produced consistently and rapidly in stock weanling rats subsisting on diets with various plant materials completely devoid of animal protein.

Methods and procedures. Black rats de-

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TABLE I. Composition of Diets.

Foodstuff		Addition to basal diet* (parts)	Protein in diet (%)
Cornmeal, white, American†		74	7.9
" " African ("Mealie meal")‡		"	6.8
Rice, white, uncoated		"	6.0
Cassava§		"	.8
Wheat flour, white‖	Wheat diet	55.65	7.8
Glucose		18.35	
Casein¶	choline deficient diet	9	8.1
Glucose		67	
Salts		1	

* See text.

† Bolted, water ground, "Indian Head" Wilkins-Rogers Milling Co., Washington, D. C.

‡ Supplied through the courtesy of Dr. R. J. Smit, Pretoria, Union of South Africa. This refined cornmeal is the type used as staple food of many natives of this area.

§ A refined flour supplied through the courtesy of Dr. R. J. Smit.

‖ Bleached, brominated, unenriched. Russell-Miller Milling Co., Minneapolis.

¶ Casein, plain, untreated, General Biochemicals, Inc.

rived originally from a mixed stock and bred at random have been used. Breeding animals were maintained on Rockland "D-free" pellets supplemented twice weekly with fresh milk. At weaning (21 days), young rats were placed on one of a number of diets containing different plant materials. Table I shows the type and source of plant foodstuffs and amount of each in the diet. Common to all diets were the following ingredients: salts (Hubbell, Mendel, and Wakeman) 3; Crisco 15; 5% cod liver oil in corn oil 2; vitaminized glucose(1) 3. Nitrogen was determined in each of the protein-containing foodstuffs by micro-Kjeldahl. To convert to protein the nitrogen values were multiplied by 6.25 for casein, corn and cassava, 5.95 for rice, and 5.7 for white flour(2). Procedures for chemical and histological studies have been previously reported(1).

Results. Comparison of various plant materials. It will be noted from Table II that about 80% of the stock weanling rats on American cornmeal diet developed portal type of fatty liver within 28 days. This type of fatty liver also occurred in rats ingesting the diets with other plant materials. In only one of 108 rats was the fat located predominantly in the central portion of the lobule. This sole exception was one of the 9 rats on cassava diet. No difference between sexes was apparent, hence data for both sexes are combined in Table II. In these experiments, rats from about 30 different litters were used, so that the results do not reflect a possible idiosyncrasy of a few litters, but rather indicate a general response to the test diets.

Chemically determined concentration of lipid in livers, and growth of animals on plant rations are given in Table III. Corn meal and rice diets caused the highest concentrations of fat; these were followed (in order of decreasing lipid content) by "mealie meal" cassava, and white flour. Growth was best on the rice diet, followed by corn meal, "mealie meal", flour and cassava. Cassava with its low protein content caused immediate weight loss, but the other diets allowed some growth. Data are included for rats on American cornmeal diet which were killed after 28 days, because these were the animals whose microscopic

TABLE II. Microscopic Distribution of Hepatic Lipid of Rats Subsisting on Diets Containing Various Plant Foodstuffs. (Sudan IV stain.)

Diet	Days on diet	No. of rats	Site of lipid in lobule — Portal — Definite	Portal — Tendency*	Diffuse†	Little or no fat
American cornmeal	28	62	29	20	9	4
"Mealie meal"	21–28	8	5	1	2	0
Rice	21	21	11	8	2	0
Cassava	18–23	9	6	2	0	0
Wheat	21–28	8	4	0	4	0
Totals		108	55	31	17	4

* Tendency = fat distributed throughout lobule but with largest droplets and greatest density occurring in the portal area.

† Diffuse = fat distributed throughout lobule without obvious localization in droplet size or density; usually, but not always, noted in very fatty livers.

TABLE III. Lipid Accumulation and Growth of Rats Subsisting on Diets Containing Various Plant Foodstuffs.

Diet	American corn meal				Rice		Cassava		"Mealie meal"		White flour	
Days on diet	21		28		21		18-23		21-28		21-28	
Sex	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀
Liver lipids:												
No. of rats	4	5	20	14	4	10	4	5	2	6	2	8
Avg, %*	9.8	14.4	12.1	15.6	14.5	14.7	7.4	7.9	12.8	10.0	5.8	5.9
Range, %	8.5-11.7	10.3-19.1	5.5-19.5	7.6-20.9	10.5-18.5	6.0-20.2	4.8-12.5	5.4-9.2	5.0-20.6	4.7-14.7	3.9-7.8	3.7-9.4
Total wt gain (g):												
No. of rats	6	16	20	14	4	10	4	5	2	6	2	8
Avg, g	6	10	11	13	13	12	−7	−7	5	3	5	6
Range, g	4-8	7-14	8-16	8-19	12-16	8-15	−5 to −10	−5 to −10	2-8	2-6	4-7	4-8

* Wet wt basis.

findings are summarized in Table II.

Females on this diet had higher lipid percentages than males (Table III). The significance of this sex difference was in question because many of these animals were not littermates, and there was considerable variation in their hepatic lipids. A subsequent experiment designed to test this point did not show a significant difference. When 14 pairs of littermate rats were analyzed after 28 days on the diet, the average lipid percentages were: males 9.6% (5.3-21.7%); females 12.6% (6.1-20.9%). Analyzed statistically by the "t" test, the difference was not significant ($P = 0.14$).

Sequential development of hepatic lipid. To study the development of the fatty liver, rats of both sexes were fed American cornmeal diet and killed after various periods (Table IV). After one week on the diet, 5 of 6 rats were found to have increased amounts of lipid in the liver, and this lipid was in the portal areas of the lobules. The concentration of lipid increased through the 12th week, but, while fat droplets were appearing throughout the lobule, the fatty change remained most severe in the portal areas. In the early weeks, the fat droplets were multiple, small, and discrete within the cells, and did not displace the nucleus. With increasing time, the droplets coalesced to form single masses within the cytoplasm. By the 12th week, very large droplets were present in the portal regions. These fatty masses filled and expanded the cells, frequently displacing the nucleus. No evidence of "fatty cysts" described by Hartroft(3) in choline-deficient rats were seen in these livers. This progressive increase in fat was associated with a very slow growth rate of about 3 g per week, average. This study raises the question of the similarity of the natural history of this type of fatty liver to the central type seen in choline deficiency. The latter, if sufficiently severe and prolonged, may develop into cirrhosis. Whether the portal type will eventually result in cirrhosis is unknown at present.

Choline deficiency and site of lipid deposition. Most of the work of others on fatty liver has been done using white rats on choline-deficient diets; such rats develop a

TABLE IV. Liver Lipid and Weight Increment Values in Rats Killed at Varying Periods on American Cornmeal Diet.

	Weeks on cornmeal diet							Weeks on stock diet
	0	1	2	3	4	8	12	2†
Liver lipids (%):*								
Avg	3.1	5.7	7.4	12.3	14.9	18.0	24.1	2.4
Range	2.5-3.4	4.5-7.9	4.9-10.4	9.1-19.1	11.3-18.2	9.3-25.5	15.2-29.5	2.0-3.0
No. of rats	6	6	8	9	4	6	8	3
Site of fat:								
Portal	0	5	7	8	4	4	8	0
Central	0	0	0	0	0	0	0	0
Diffuse	0	0	1	1	0	2	0	0
Little or no fat	6	1	0	0	0	0	0	3
Total wt gain (g):	Initial values							
Avg	30	4	6	9	11	23	36	54
Range	28-34	2-8	2-13	4-14	4-18	13-35	20-47	53-55
No. of rats	46	41	31	22	18	13	8	3

* On wet wt basis.
† Littermate rats killed after 2 wk on stock diet for comparison.

fatty liver which is central in type. Our black rats respond similarly to choline deficiency. Fourteen stock weanling rats, subsisting on choline-deficient diet (Table I) for 28 days had centrally located hepatic fat in each instance. The lipid concentration averaged 16.2% (9.5-24.6%); the rats gained an average of 47 g (18-66 g) in this period.

Discussion. As far as has been determined by histological and chemical means, portal fatty liver produced in these experiments is identical to that found in our earlier experiments with "second generation" rats. The production of this disturbance with relative ease and rapidity has several obvious advantages.

It is also of interest that the "mealie meal" and cassava which, like the corn, produced portal fatty liver, were obtained from Africa where they are staple foods of Africans among whom kwashiorkor(4,5) occurs. Likewise, the findings in our rats ingesting rice and white flour are in harmony with reports of a similar type of fatty liver which occurs in children subsisting largely on rice(6) or wheat flour (7-9) with an inadequate intake of nourishing supplementary foods.

Summary. A portal type of fatty liver has been rapidly and consistently produced in stock weanling rats by feeding diets in which the protein was exclusively of plant origin (corn, rice, wheat or cassava). This experimental lesion bears a similarity to the type of fatty liver seen in the human disease kwashiorkor. Within one week on a corn meal diet excess fat appeared in the portal region of the liver lobule and increased in amount over the 12-week period of study. Even with large concentrations of fat, the portal areas were clearly the most seriously involved.

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Chloramphenicol Resistance in *Micrococcus pyogenes*. II. Intermediary Metabolism of Sensitive and Resistant Strains.* (21417)

J. L. Padron, S. C. Smith, and H. H. Ramsey.

From the Departments of Biochemistry and Bacteriology, University of Oklahoma School of Medicine, Oklahoma City.

In a previous communication(1) it was noted that chloramphenicol resistance in *M. pyogenes* was accompanied by a decreased requirement for niacin and thiamine. This observation may be interpreted in at least two ways. First, the decreased vitamin requirements may be a reflection of vitamin synthesis by the resistant organism. Alternatively, it may be the result of a shunted metabolism in which the reactions involving the coenzymes of thiamine and niacin are no longer operable. The present communication presents preliminary evidence in favor of the second possibility.

Materials and methods. The test organisms consisted of a strain of *M. pyogenes* inhibited by 4.5 μg chloramphenicol per ml and a variant developed from it which was resistant to 1040 μg chloramphenicol per ml. Working stock cultures were carried on slopes of Bacto-A.C. broth supplemented with 2% agar. For manometric experiments, Roux bottles containing 200 ml agar were inoculated with 5 ml of a 24 hour broth culture. After incubating for 24 hours at 37°C the cultures were harvested with 10 ml of chilled 0.15 *M* phosphate buffer (pH 7.4), washed twice and resuspended in 10 ml buffer. The suspension was diluted 1:5 in buffer and 1 ml used in each Warburg vessel. All experiments were performed at 37°C with air as the gas phase and KOH in the center well; total volume of each flask was 3 ml. Lactic acid was determined by the method of Barker and Summerson(2).

Results. It has been found that chloramphenicol resistance is accompanied by a sharp drop in the rate of oxidation of pyruvate; the sensitive strain oxidizing the substrate at a rate of approximately 4 times that of the resistant strain (Fig. 1a). Further, the resistant strain has an extremely high endogenous metabolism so that, if endogenous is not corrected for, the O_2 consumption by both strains is practically identical (Fig. 1b). The nature of this high endogenous has not yet been determined, however, it is apparently not due to pyruvate or an oxidation product thereof, since addition of pyruvate caused only a minor change in the rate of respiration. As noted by Reiner and Spiegelman(3), preferential use of reserve cellular substances over that of an exogenous substrate would result in no change in the endogenous rate of metabolism upon addition of the exogenous sub-

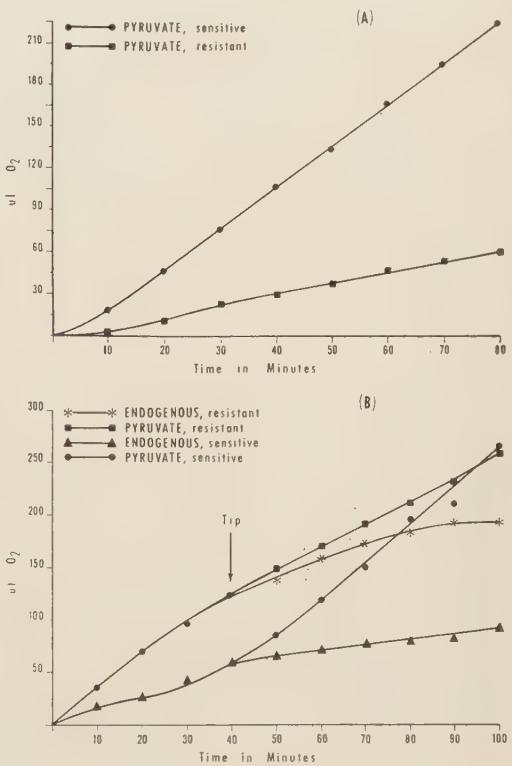

FIG. 1. Oxidation of pyruvate by sensitive and resistant strains. (A) Endogenous respiration subtracted; (B) Endogenous respiration shown. Dry wt of cells, 14 mg. Pyruvate concentration, .01 mM.

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TABLE I. Oxidation of Glucose, Acetate and Pyruvate, and Effect of Chloramphenicol (CM) thereon.

	μl O_2 consumed*			
	Resistant		Sensitive	
Substrate, .01 mM	No CM	200 μg CM	No CM	200 μg CM
Glucose	344	328	311	326
Pyruvate	8	12	248	241
Acetate	0	0	108	112

* Observation period 80 min. Corrected for endogenous. Dry wt of cells, 14 mg.

strate. Inability of the resistant strain to utilize pyruvate as a substrate was confirmed by measurement of dehydrogenase activity using the conventional Thunberg technic.

Acetate, like pyruvate, is not oxidized by the resistant organism to any significant extent, whereas glucose is oxidized at an equal rate by both strains (Table I). This would indicate that the metabolic block occurs at the pyruvate-acetate level. If this block involved the loss of an enzyme or coenzyme necessary for decarboxylating pyruvate and its subsequent utilization in the Krebs cycle, it might be expected that pyruvate would serve as a hydrogen acceptor and lactic acid would accumulate in the medium. In order to test this possibility, equal aliquots of both strains were incubated for two hours in the presence of glucose and lactic acid determined. In replicate experiments, it has been found that the resistant organism produces approximately 2 times as much lactic acid (1 mole/mole glucose) as does the sensitive organism (.5 mole/mole glucose).

Attempts to reconstitute the pyruvate oxidase system of intact cells of the resistant strain by addition of Mg^{++}, thiamine, thiamine pyrophosphate, DPN, niacin, nicotinamide, α-lipoic acid, coenzyme A and various combinations of these have been without success. Experiments utilizing cell-free extracts are currently under way.

Rosanoff and Sevag(4) have noted that resistance to streptomycin in *E. coli* is also accompanied by a loss in the ability to oxidize pyruvate and a concomitant increase in lactic acid accumulation. Whether or not the mechanism reported by these authors and that noted herein is the same cannot at present be ascertained, since in neither instance has the metabolic lesion been located. However, Oginsky *et al.*(5) and Smith *et al.*(6) have suggested that streptomycin inhibits the condensation of pyruvate and oxalacetate and thereby prevents further oxidation of pyruvate. The possibility that chloramphenicol functions in a similar manner cannot be excluded, particularly since the resistant organism also fails to oxidize acetate. However, it should be noted that 200 μg of chloramphenicol fails to suppress endogenous respiration of either strain and also fails to inhibit the oxidation of glucose, pyruvate and acetate by the sensitive strain (Table I).

Summary. Resistance to chloramphenicol in *M. pyogenes* is accompanied by 1) high endogenous respiration, 2) loss of ability to oxidize pyruvate and acetate, and 3) increased formation of lactic acid from glucose. The antibiotic exerts no depressant effect on any of these reactions carried out by a strain sensitive to the drug. Attempts to reconstitute the pyruvate oxidase system of intact cells resistant to the antibiotic have been unsuccessful.

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Liver Circulation and Common Duct Obstruction. (21418)

N. C. Jefferson, H. L. Popper, and H. Necheles.*
(With the technical assistance of Mr. Charles Roddick.)
From the Department of Gastro-Intestinal Research, Medical Research Institute of Michael Reese Hospital, Chicago, Ill.

We have reported results of experiments in which there was interference with portal circulation(1-4). In continuation of this work we have tried to find what would happen when obstruction of the common bile duct would be added to excision of the hepatic artery or to obstruction of the portal vein.

Methods. Thirty-eight healthy mongrel dogs of both sexes were used, weighing 8 to 16 kg, and aseptic operations were performed under pentobarbital anesthesia. Penicillin and streptomycin were administered for the first 3-5 postoperative days and, whenever necessary, intravenous glucose solution immediately following the operation. The hepatic artery was excised from its origin up to the liver, where all its branches were ligated and cut(5). The portal vein was obstructed by 2 incomplete ligatures, about 1 cm apart, which as we have reported(6), produced complete occlusion of the vein within a few days. The common bile duct was cut between ligatures just below the confluence of the hepatic ducts. In all animals cholecystectomy was performed.

Results and discussion. Table I. While mortality following excision of the hepatic artery alone was 20% (3 out of 15, Group F), and mortality following division of the common duct alone was 18% (2 out of 11, Group F), mortality of the combined procedure was 77% (7 out of 9, Group A). It is noteworthy that 4 fatalities of the last group showed foamy livers. While antibiotics prevent anaerobic liver necrosis following excision of the hepatic artery, additional biliary obstruction seems to cause a situation where antibiotics are unable to cope with the infection. This may be explained by our recent finding, that excision of the hepatic artery combined with obstruction of the common bile duct leads to an impairment in the intrahepatic portal circulation. The complete loss of the hepatic arterial blood supply and the gradually diminishing portal blood supply, seem frequently not compatible with survival of the liver.

The experiments in which the 2 procedures were separated (Group B and C) showed the following: when division of the common bile duct was done first and excision of the hepatic artery at a second stage, mortality was 85% (6 out of 7, Group C), but if excision of the hepatic artery was done first and division of the common bile duct later, mortality was 50% (7 out of 14, Group B). Separation of the 2 procedures with excision of the artery first seems to give a higher survival rate, possibly due to the fact that increased arterial blood supply to the liver through the phrenic arteries is already established at the time of the decrease in portal blood supply following biliary obstruction.

Of 4 dogs (Group E and 2 dogs of Group A) in which in several stages the hepatic artery had been excised, the portal vein obstructed and the common bile duct ligated and cut, one dog survived. This shows that even this combination of procedures will occasionally be compatible with survival. Of course, all dogs with ligation of the common bile duct die after a few weeks' time.

The mortality of the dogs in Group D, in which division of the common bile duct was combined with obstruction of the portal vein, was 17% (1 out of 6), which is practically identical with the mortality in the control series with obstruction of the common duct only (18%). Since the main oxygen supply of the liver through the hepatic artery remained intact in these experiments, biliary obstruction had no immediate deleterious effect.

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TABLE I.

Group	First operation	No. of dogs: Used	No. of dogs: Died (days p.o.)	Second operation: Days after 1st operation	Second operation: Procedure	No. of dogs: Died (days p.o.)	No. of dogs: Survived
A	Excision of hepatic artery and section of common bile duct	9	7 (1-7)	21	Obstruction of portal vein	1 (1) 1 (6)*	0
B	Excision of hepatic artery	14	3	7–11	Section of common bile duct	4 (1–6)	7
C	Section of common bile duct	7	1	4–21	Excision of hepatic artery	5 (1–5)	1
D	Obstruction of portal vein and section of common bile duct	6	1	14–21	*Idem*	3 (1)	2
E	Excision of hepatic artery and obstruction of portal vein	2	0	75 and 120	Obstruction of common bile duct	1 (2) 1 (3)	0
F	Excision of hepatic artery	15	3				13
	Section of common bile duct	11	2				9

* With perforated duodenal ulcer.

Summary. 1. Excision of the hepatic artery combined with obstruction of the common bile duct has a high mortality. Administration of antibiotics was not able to prevent anaerobic growth and liver necrosis in some of these dogs. 2. If the above 2 procedures are performed in 2 stages, mortality is somewhat lower if excision of the hepatic artery is done first and obstruction of the common bile duct later, while performance of these procedures in reversed sequence has a very high mortality. 3. Obstruction of the portal vein and of the common bile duct, performed in one stage, is well tolerated. 4. The combination of excision of the hepatic artery and obstruction of the portal vein and of the common bile duct will occasionally be survived by the dog.

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Preservation at Subzero Temperatures of Mouse Fibroblasts (Strain L) and Human Epithelial Cells (Strain HeLa).* (21419)

William F. Scherer and Alicia C. Hoogasian.

From Department of Bacteriology, and Immunology, University of Minnesota, Minneapolis.

Strains of animal cells maintained *in vitro* by continuous cultivation are currently employed for research in many fields of experimental biology and medicine. Use of such strains may be limited, however, by their tendency during long periods of continuous cultivation to gain or to lose such properties as malignancy(1,2) and by the labor required to

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feed and subculture the cells during periods when they are not needed for experiments. Since storage of cells by freezing should circumvent these difficulties, a study has been carried out to learn whether cells of 2 established strains can be preserved at subzero temperatures. This paper reports findings concerned with storage of Earle's mouse fibroblasts, strain L(1), and Gey's human epithelial cancer cells, strain HeLa(3). Five variables were studied: composition of suspending medium; rate of freezing; temperature of storage; duration of storage; and rate of thawing.

Materials and methods. Cells. Strain L cells derived from mouse subcutaneous tissue and strain HeLa cells derived from a human epidermoid cervical carcinoma have been maintained in this laboratory by continuous cultivation(4-6) since 1951 and 1952, respectively. *Media for storage of cells.* Cells for storage at subzero temperatures were suspended in the following media: a) a maintenance solution (MS) for animal cells(4,6); b) horse serum (HoS) for strain L cells, or adult human serum (HuS) for strain HeLa cells; sterile sera obtained as described(4,6); c) glycerol† diluted with MS; d) glycerol diluted in Hanks' balanced salt solution(7); e) glycerol diluted with HoS or HuS; and f) dextrose 5 g % in glass-distilled water. Media 1-5 were used at pH 7.4-7.6; medium 6 at pH 6.6, or at pH 7.2-7.4 after addition of 1.4% sodium bicarbonate in glass-distilled water. Media a, c, d and f were sterilized by autoclaving at 115°C for 10 min. *Preparation of cell suspension for storage.* Strain L and strain HeLa cells were cultivated on walls of square screw-capped "Neutraglas" bottles of 200 ml capacity. They were removed from the glass by a glass or platinum spatula or by a stream of medium blown from a 5 or 10 ml serologic pipette. Forceful pipetting of the cells provided an evenly dispersed suspension. A 1-ml aliquot of the suspension was centrifuged at 1500 or 2000 rpm for 5 to 10 minutes, and the supernatant fluid removed and replaced by 1 or 2 ml of 4 or 5% trypsin‡ in MS. After incubation for 20-30 minutes at 36°C, the cells in trypsin were dispersed by repeated forceful expulsion from a capillary pipette and transferred to a hemocytometer for counting(5). After calculating the population density of the original suspension, aliquots containing known numbers of cells were centrifuged and the supernatant fluids discarded. The cells were resuspended in the medium selected for study and transferred in 1 or 2 ml amounts to 2 ml ampules, which were sealed by heat. The number of cells per ampule varied for each experiment and ranged from 100,000 to 5,000,000. *Freezing and storage of cells.* Four procedures were used: a) sudden immersion of ampules in a mixture of 95% ethanol and solid CO_2 at –70°C resulting in freezing of contents within 3-5 minutes, followed by storage at –60° to –70°C over solid CO_2; b) immersion of ampules in 95% ethanol to which solid CO_2 was added to depress the temperature to –70°C in 1-1.5 hours, followed by storage at –60° to –70° C: this method was similar to that employed by Smith(8) for freezing sperm and granulosa cells; c) freezing at –70°C in 3-5 minutes, as in procedure a), with storage at –20°C in an electric refrigerator; and d) transfer of ampules from room temperature to a –20°C refrigerator without preliminary freezing.

Thawing and revival of cells. Frozen cell suspensions in ampules were thawed by: a) transfer from storage cabinet to a 37°C water bath, which resulted in thawing within 1-2 min.; b) transfer from –60° to –70°C to an ethanol-CO_2 bath at –70°C, with the temperature of the mixture increased gradually by addition of warm water over 30-60 minutes until the cell suspension was thawed; c) transfer from –60° to –70°C to a refrigerator at –5° to –8°C for 15 minutes, then to a +5°C refrigerator for 15 minutes and to room temperature (25-29°C) for 1-3 minutes. The last step was omitted with the 20% glycerol medium, which became liquid at +5°C external to ampule. After thawing, each cell suspension was transferred to a 16 x 125 mm screw-capped tube or to a stoppered 13 x 100 mm tube. The ampule was rinsed once with 1-2 ml of 5% serum (HoS for strain L and HuS for strain HeLa) in Hanks' salt solution, and this fluid added to the tube. After centri-

† Merck, reagent grade.

‡ Difco trypsin 1:250.

TABLE I. Survival at −60°C to −70°C of Mouse Fibroblasts, Strain L, and Human Epithelial Cells, Strain HeLa: Effects of Glycerol Concentration and Rate of Freezing.

		Cell survival (%) after storage for 2–16 days			
		Frozen in 5 min.; thawed in 2 min.		Frozen in 1.5 hr; thawed in 2 min.	
Cell strain	Glycerol, %	Glyc. in MS	Glyc. in serum*	Glyc. in MS	Glyc. in serum*
L	0	0	0–14	0	0–14
	2	3–10	12–28	11–21	3–30
	5	10–19	15–50	11–45	20–76
	7	3		+†	
	10	3–17	6–20	6–44	3–37
	15			+†	0
	30	0		0	0
	60	0		0	
	100	0		0	
HeLa	0	0	1– 3		
	4	2	1		
	5	11–22	9–15	1– 4	13–42
	7	12	6		
	15	21–65	35–67	13–25	24–59
	20	25–67	16–81	21–29	18–59
	30	33–67	11–62	31–59	18–40
	50	2–45	11–41	13–23	15–22

* Horse serum for L strain, human serum for HeLa strain.

† + = Cells survived but not counted.

fugation at 1500-2000 rpm for 5-10 minutes and removal of supernatant fluid, the cells were resuspended in 0.5-1.0 ml of 40% serum in Hanks' solution. The 16 x 125 mm tubes were slanted at 36°C, with caps tightened. Initially, the cells in the 13 x 100 mm tube were transferred to a 16 x 125 mm tube or to a Porter flask for convenient incubation; later this transfer was omitted because of occasional loss of cells. After 1 day's incubation, the cells were observed microscopically and the supernatant fluid removed from each tube. Enumeration of cells treated with trypsin was performed as described for cell suspensions before freezing. Although cell debris was present in occasional cultures of thawed cells before treatment with trypsin, little was seen in the hemocytometer and it was not difficult to count intact cells.

Results. Effect of medium composition. Survival rates after fast or slow freezing of mouse fibroblasts, strain L, suspended in MS or horse serum containing varying concentrations of glycerol and stored at −60° to −70°C are presented in Table I. With both freezing methods, cells failed to survive when suspended in MS alone, or in MS with glycerol 30%, 60%, or 100% (w/v). From 3-45% of the cells were recovered when suspended in MS with 2-10% glycerol. Strain L cells were preserved in horse serum with and without glycerol. Recovery rates were better, however, with glycerol 2-10% than with 0, 15 or 30%. The results with strain HeLa cells (Table I) show: a) that cells survived storage in HuS alone but not in MS; b) that cells were preserved in glycerol-MS or glycerol-HuS mixtures that contained 4-50% glycerol; and c) that the survival rates were usually higher at glycerol concentrations of 15-30% than at lower or higher levels of glycerol employed. Contrasting levels of glycerol optimal for survival of strain L and strain HeLa cells may be noted. Survival of both cell types in serum alone but not in undiluted MS suggests that serum is the superior diluent for glycerol. These studies are being continued to compare cell survival rates after months or years of storage. The data from experiments carried out to learn the survival rates of strain L and strain HeLa cells in mixtures of glycerol with Hanks' balanced salt solution, and in dextrose 5 g % in water, are tabulated in Table II. For the short duration of these experiments, the two cell types survived equally well in glycerol-Hanks' solution or glycerol-MS solution, but comparatively poorly in 5% dextrose solution.

Effect of freezing rate. A comparison of survival rates of cells frozen quickly or slowly to −60° to −70°C (Table I) revealed no consistent differences for strain HeLa suspended in glycerol-MS or glycerol-HuS mixtures; for strain L, survival rates were occasionally higher after the slow-freeze method. Long term experiments in progress may evaluate the two freezing methods more precisely.

Effect of storage temperature. Cells were preserved at −60° to −70°C on solid CO_2 (Table I); cells did not survive freezing to −70°C and subsequent storage at −20°C in an electric refrigerator (Table III). Cells frozen by direct transfer to −20°C usually survived, although less well than after freezing and storage at −70°C (Table III).

Effect of duration of storage. To date

TABLE II. Effects of Medium Composition upon Survival of Cells Kept in Storage at –60° to –70°C.

Strain	Cells, No./ampule (× 1000)	Days frozen	Surviving cells (× 1000)		
			Glycerol* in MS	Glycerol* in Hanks' sol.	5% dextrose in water
L	918	1	342	328	
		2	478	430	57
	550	3	346	286	40
		4	346	238	29
	554	1	145	136	17
		2	179	208	10
HeLa	1400	4	522	734	
		11	935	944	
		14	721	820	
	1380	7	644	653	0
		8	490	571	0
		13	540	466	0
	880	6	531†		8
		7	650		9
		8	720		12
	556	5			0‡
		8			0
	456	5	114	92	

* For strain L, glycerol 5%; for strain HeLa, glycerol 20%.
† Frozen in glycerol and serum.
‡ Dextrose in water at pH 6.6 for this experiment; at pH 7.4 for other experiments.

(Sept. 1954), strain L cells have been stored successfully at –60° to –70°C for approximately 6 months in 5% glycerol in MS and for 5 months in 5% glycerol in HoS (Table IV); strain HeLa cells have survived in 30% glycerol in MS for 7 months (Table IV). It is important to note that cells stored for short periods of time survived better at –60° to –70°C than at –20°C (Table III). Experiments are in progress to study long term preservation of cells at –20° and at –70°C.

Effect of thawing rate. Cells were thawed rapidly (within 2 minutes), slowly (in an ethanol-CO_2 mixture within 30-60 minutes), or in 3 stages (15 minutes at –5 to –8°C, 15 minutes at +5°C and several minutes at 25-29°C). Comparison of the numbers of cells that survived these methods of thawing (Table V) shows that usually more cells survived rapid thawing than survived the 2 slower procedures. This apparent damaging effect of slower thawing may be a factor in the total destruction of cells observed after freezing to –70°C and storage at –20°C, since a slow partial thawing of cells occurred upon transfer from the lower to the higher temperature.

Comparison of cells before and after storage at –60° to –70°C. Three attributes of strain L and of strain HeLa cells were compared before and after freezing and thawing: a) morphology, b) growth rate, and c) susceptibility to viruses. The morphology of the cells after storage to date, has been indistinguishable from that observed before storage. Strain L and strain HeLa cells preserved at –60° to –70°C grew as rapidly as unfrozen cells. Both cell types assumed their normal shape, size and appearance, and showed mitoses 48 hours after thawing and incubation at 36°C. To test the susceptibility of stored cells to viruses, cells from 1-3 ampules were cultivated for 3-7 days in 16 x 125 mm test tubes, in Porter flasks or in flat-sided test tubes, until sufficient cells were obtained to inoculate square 200 ml bottles. Cells from the bottle cultures were then used to make tube cultures for inoculation with virus. Strain HeLa cells derived from cells stored at –60° to –70°C were as susceptible as unfrozen cells to the viruses of poliomyelitis (Types 1, 2 and 3), herpes simplex, vaccinia, and Eastern equine encephalomyelitis; similarly, progeny of stored strain L cells showed unaltered susceptibility to the viruses of Eastern equine encephalomyelitis and her-

TABLE III. Effects of Temperature of Storage upon Survival of Cells.

Cell strain	Suspending liquid: Glycerol, %	Suspending liquid: Diluent	Cells: No./ampule (× 1000)	Cells: Days frozen	Cells surviving (× 1000): Freezing* & storage at −60 to −70°C†	Cells surviving (× 1000): Freezing & storage at −20°C†	Cells surviving (× 1000): Freezing in 5 min. to −70°C & storage at −20°C†
L	5	MS	918	1	342	107	
				2	478	162	
	"	HoS	550	3	358	67	
	"	"	554	1	240	200	
				2	189	82	
	"	MS	2260	2	+‡		0
				13	+		0
				33	163		0
	"	HoS	4000	2	377		0
			1300	2	159		0
			430	2	60		0
			143	2	15		0
HeLa	20	HuS	1380	7		173	
				8		43	
				13		0	
	"	"	880	6	531	272	
				7	650	378	
				8	720	182	
	"	"	636	19	68	59	
	"	MS	1420	4	522		0
				11	935		0
				14	721		0
	"	"	1380	7	644		0
				8	490		0
				13	540		0
	"	"	n.c.§	1	444		0
				5	313		0
	"	"	n.c.	6	435		0

* Time of freezing was either 1.5 hr or 5 min. for strain L and 5 min. for strain HeLa.
† Thawed in 2 min. ‡ + = Cells survived but not counted. § n.c. = Not counted.

pes simplex.

Discussion. A variety of living animal cells and tissues, *e.g.,* normal mammalian skin (9,15), thyroid(10), kidney(11), ovary(8), erythrocytes(12), spermatozoa(13) and many mammalian tumors(14-18), have been preserved by freezing and storage at subzero temperatures. The methods employed to achieve successful preservation of the cells have often differed in: a) composition of medium used to suspend the cells; b) rate of freezing; c) temperature of storage; d) duration of storage, and e) rate of thawing. Glycerol has been added to the suspending medium for spermatozoa(13), ovarian granulosa cells(8), erythrocytes(12), and certain tumor tissues(17). Various combinations and methods of fast and slow freezing and fast and slow thawing have been evaluated for selected cells or tissues. Since the reported experiments did not indicate that any one preservation procedure might be generally optimal, it was essential for this study of the subzero preservation of mouse fibroblasts (strain L) and human epithelial cancer cells (strain HeLa) to evaluate the effects of the variables mentioned.

The results show that strain L and strain HeLa cells can be preserved in solutions of glycerol in serum, MS, or Hanks' solution much better than in undiluted serum or in 5% dextrose in water. The proportion of glycerol optimal for survival of strain HeLa cells was higher than for strain L cells. Neither cell type survived freezing and thawing in undiluted MS. The preservation of strain L cells was slightly better when freezing was performed in 1-1.5 hours than in 3-5 minutes; strain HeLa cells withstood both methods of freezing equally well. Storage of cells at −60° to −70°C has been more successful than at

TABLE IV. Long-Term Storage at −60° to −70° of Mouse Fibroblasts, Strain L, and Human Epithelial Cells, Strain HeLa.

Cell strain	Suspending liquid		Cells		Cell survival (%)	
	Glycerol, %	Diluent	No./ampule (× 10^6)	Days in storage	Frozen in 5 min., thawed in 2 min.	Frozen in 1.5 hr, thawed in 2 min.
L	5	MS	2.26	23	25	
				33	7	
				99	6	
				176	19	
				177	20	
	5	HoS	1.60	65	5	12
				141	28	23
				142	33	8
HeLa	30	MS	3.54	29	36	
				78	30	
				99	28	
				134	23	
				210	3	
			1.80	43	32	
				58	30	
				93	22	
				113	20	
				121	21	
				149	16	
				225	7	
			1.78	36	53	
				45	49	
				80	42	
				100	37	
				136	25	
				212	3	

−20°C. Thawing over periods of 1-2 minutes was usually superior to thawing over 30-60 minutes. Strain L cells have now been stored at −60° to −70°C for 6 months and strain HeLa cells for 7 months. The variations observed in percentages of strain L cells recovered from single ampules at intervals of 23-177 days after freezing (Table IV) could theoretically have been caused by: a) uneven distribution of cells to the ampules; b) variation in damage to cells in different ampules during freezing or thawing; or c) loss of cells from certain ampules during transfer of the thawed cell suspension to a culture tube. The latter factor was minimized by thoroughly rinsing each ampule and adding the fluid to the cell suspension already transferred to a culture tube for centrifugation. Possibilities a) and b) are being studied further.

The results of these experiments make the preservation of strain L and strain HeLa cells of practical significance for 5 reasons: a) the labor required for maintenance of these cell strains can be decreased substantially since cells can be frozen when not needed for experiments; b) the stability of these strains can be better assured; by their storage in the frozen state, the intervals between transfer of cells can be prolonged to reduce the possibility of a change in properties of the cells resulting from continuous cultivation; c) a single stock of frozen cells can be employed to provide similar populations of cells for experiments performed at different times; d) shipment of cells can be facilitated by packaging with solid CO_2; e) if, in the future, the properties of strain L or strain HeLa become changed, *e.g.*, with respect to their morphology, physiology, virus susceptibility or malignancy, the altered cells could be compared with the parent cells that have been preserved at subzero temperatures. The results of this study and of studies with other cells(8-18) suggest that cells of other strains could perhaps be preserved similarly in solutions of glycerol. Strains of normal cells might then be preserved without danger of their undergoing the malignant transformations that

TABLE V. Effects of Rate of Thawing upon Survival of Cells.

Cell strain	Suspending liquid: Glycerol, %	Suspending liquid: Diluent	Cells: No./ampule (× 1000)	Cells: Days frozen	Cells × 1000 surviving freezing,* storage at −60° to −70°C, thawing within: 2 min.	30 min.: Ethanol-CO_2	30 min.: Refrigerator
L	5	MS	918	1	342	40	
				2	478	432	
	"	HoS	550	3	358	179	
	"	"	554	1	240	168	113
				2	189	84	99
HeLa	20	MS	1420	4	522		32
				11	935		266
				14	721		454
	"	"	1380	7	644		185
				8	490		185
				13	540		47
	"	"	n.c.†	1	444		10
				5	313		175
	"	"	n.c.	6	435		0
	"	HuS	880	6	531	21	9
				7	650		14
				8	720	4	8

* Time of freezing was either 1.5 hr or 5 min. for strain L and 5 min. for strain HeLa.
† n.c. = Not counted.

sometimes accompany continuous cultivation of cells *in vitro*; normal cells so maintained a) could be compared, eventually, with their malignant derivatives, and b) might be useful for grafting purposes.

Summary. Mouse fibroblasts of strain L (Earle) and human epithelial cells of strain HeLa (Gey) have been preserved successfully at −60° to −70°C for 6 and 7 months, respectively. Glycerol in the suspending liquid greatly increased the percentage of surviving cells. Solutions of glycerol (5% for strain L and 20% or 30% for strain HeLa) in a solution for maintenance of animal cells (MS), in balanced salt solution, or in serum (horse serum for strain L cells, adult human serum for strain HeLa cells), were of approximately equal value for the periods of preservation studied. The cells survived storage poorly at −60° to −70°C when suspended in undiluted serum or in 5% dextrose in water. Strain L cells were preserved in slightly greater numbers after freezing over a period of 1-1.5 hours than after freezing in 3-5 minutes; strain HeLa cells survived both freezing procedures equally well. Better preservation of both cell types resulted from storage at −60° to −70°C than at −20°C, and from thawing in 1-2 min. rather than in 30-60 minutes. The value of low temperature preservation for handling and maintenance of strain L and strain HeLa cells and for preservation of strain stability is discussed.

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Plasma Sympathin Concentrations of Dogs.* (21420)

JACK W. MILLER† AND HENRY W. ELLIOTT.

From the Department of Pharmacology and Experimental Therapeutics, University of California School of Medicine, and College of Dentistry, San Francisco.

There are a variety of methods available for the estimation of epinephrine and norepinephrine, most of which lack either the sensitivity or specificity necessary for application to plasma concentrations. Weil-Malherbe and Bone(1) have reported a sensitive fluorimetric method for the estimation of epinephrine-like substances in human plasma, which originally did not differentiate between epinephrine and norepinephrine. More recently these authors (2) presented evidence for the specificity of their method and introduced filter technics making it possible to estimate circulating concentrations of both epinephrine and norepinephrine.

This report concerns the use of their earlier method for the estimation of plasma sympathin (epinephrine plus norepinephrine) and the rate of disappearance of intravenous epinephrine in the dog.

Methods. Plasma sympathin concentrations were estimated according to the method of Weil-Malherbe and Bone(1), using a Coleman photofluorimeter equipped with PC_2 and B_3 Coleman filters. The fluorescence intensity of norepinephrine under these conditions was 1/5 that of epinephrine. The method must be followed exactly for best results and all precautions taken to avoid the introduction of extraneous fluorescent substances. For instance, Merck aluminum oxide proved unsatisfactory for absorption columns because fluorescent impurities could not be removed by washing. Aluminum oxide produced by British Drug Houses was used successfully. Ethylenediamine may be another source of error since it becomes fluorescent on standing and must be periodically redistilled. Blood glucose concentrations were estimated by adapting and combining the anthrone methods of Durham, *et al.*(3) and Loewus(4). The animals used were untrained mongrel dogs (8-14 kg) of both sexes. Blood was withdrawn from the cubital or saphenous veins of unanesthetized animals. Estimations on vagotomized dogs which received intravenous epinephrine were made during Stage III pentobarbital anesthesia. In these animals blood pressure in the carotid artery was measured with a mercury manometer, and injections were made into the left femoral vein and blood samples taken from the right vein.

Results. Table I shows the concentrations of plasma sympathin and blood glucose of untrained, unanesthetized dogs. These values are reported in terms of micrograms of epinephrine/liter of plasma, and represent an

TABLE I. Normal Control Levels of Plasma Sympathin and Blood Glucose of 8 Unanesthetized, Untrained Mongrel Dogs of Both Sexes, Arranged in Order of Increasing Sympathin Values.

	Plasma sympathin, μg/l	Blood glucose, mg %
	11	84
	15	67
	21	88
	29	83
	35	76
	40	70
	53	119
	64	111
Mean	33.5	87.2

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† Present address, Department of Pharmacology, University of Wisconsin School of Medicine, Madison.

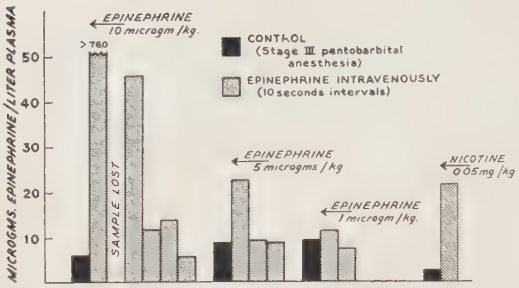

FIG. 1. Effect of various intravenous doses of epinephrine and of nicotine HCl on plasma sympathin levels of dogs anesthetized with pentobarbital.

equivalence of fluorescence only. A slight hyperglycemia was present in the 2 dogs with the highest sympathin levels, which is consistent with the hyperglycemic actions of epinephrine. These values indicate that the sympathin level in the untrained dog is roughly 10 times higher than in man(1).

Fig. 1 shows the effect of various intravenous doses of epinephrine on the sympathin levels during Stage III pentobarbital anesthesia. In this stage of anesthesia, the plasma sympathin levels were reduced to about thirty percent of the mean value for unanesthetized animals (Table I), and agree with the findings (40% reduction) of Weil-Malherbe and Bone(1) for humans. After 10 μg/kg of epinephrine, the plasma levels returned to control values after about one minute. This dose produced a marked pressor action, which paralleled the elevated plasma sympathin concentrations. Further, the sympathin level was elevated during the pressor stage following 0.05 mg/kg of nicotine HCl. The method is sensitive enough to detect plasma levels as low as 1 μg/liter or a sudden injection or secretion of 1 μg/kg of epinephrine.

Discussion. The decrease in plasma sympathin during pentobarbital anesthesia could be explained by (a) a partial depletion of the adrenal gland of epinephrine and norepinephrine during induction, (b) a diminished central stimulation of the gland or (c) a decreased production of the amines. Lund(5) has indicated that the epinephrine and norepinephrine levels of dog adrenal glands are probably reduced by pentobarbital anesthesia. This could indicate either a decreased production of these agents, or an increased secretion which would result in their partial depletion as during ether anesthesia(6). Since hyperglycemia (epinephrine release) is not characteristic of barbiturate anesthesia in dogs (7), this is evidence against depletion by an increased secretion.

The rapid disappearance of intravenous epinephrine from the plasma indicates that epinephrine was either (a) absorbed by tissues or red blood cells, (b) rapidly metabolized or (c) excreted. Since the plasma level returned to normal after the initial rise following epinephrine administration, reversible storage by tissues seems unlikely. The method is specific for adrenochrome, but there is no evidence for circulating levels of this possible metabolic product(8). Evidence for the urinary excretion by rats of epinephrine and various deaminated products has been presented. Schayer *et al.*(9) and Clark and Drell(10) have found that epinephrine glucuronide is also excreted. Because of the specificity of the present method(1), these urinary metabolites would probably not be detected if present in plasma, and could therefore account for the disappearance of intravenous epinephrine.

Summary. Plasma sympathin concentrations of dogs were reduced approximately 30% of unanesthetized values during pentobarbital anesthesia, and intravenous epinephrine rapidly disappears from the plasma, paralleling its pressor action.

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Pool Systems in Iron Metabolism; with Special Reference to Polycythemia Vera.* (21421)

LENA SHARNEY, LAWRENCE SCHWARTZ, LOUIS R. WASSERMAN, SHIRLEY PORT, AND DOROTHY LEAVITT.

From the Departments of Hematology, Physics and Medicine, Mount Sinai Hospital, New York City.

Previous reports(1,2) concerning the disappearance of a tracer dose of radioactive iron from the plasma implied a single plasma iron pool with no significant return of radioiron to the plasma during the experimental period of one to 2 hours. Assuming instantaneous mixing following the injection of ferric 59-beta-1 globulinate, as well as a constant relative disappearance rate, the theoretical data could be represented by a single exponential function, *i.e.*, a straight line on semi-log paper. Such a first approximation fitted the experimental data well because of the large errors involved in electroplating(1-3) and the relatively insensitive counting technics employed(1-3).

Two considerations prompted reevaluation of the above concepts. Firstly, utilizing improved counting technics it was noted that the experimental data were no longer approximated by a straight line when plotted on semi-log paper. Re-examination of some of the published data in earlier reports reveals similar findings(1,4). Secondly, the application of the previous approach to cases of polycythemia vera led to excessively high hemoglobin renewal rates and consequent shortened red cell survival times(1). These findings were not in accord with data obtained by other methods (5-7), nor with present basic concepts of the patho-physiology of this disorder(8). It will be demonstrated that the experimental data can be more closely approximated by the sum of 2 exponential functions, thus requiring a more complex system than the one-pool model implied by the single exponential function.

Fig. 1a represents experimental data from a case of polycythemia vera plotted on a semi-log scale, for the first hour of Fe^{59} disappearance from the plasma. The initial part of the experimental curve is approximated by a straight line from which the half time of disappearance ($T\frac{1}{2}$) is found to be 10 minutes with a rate constant, k, equal to 0.069/minute. After 30 minutes however the points begin to deviate significantly from the straight line.

Fig. 1b represents the experimental data from the same case plotted on a semi-log scale for a 6-hour period. These experimental points are approximated by the sum of 2 exponential functions:

$$\frac{q_1}{q_0} = a_1 e^{-\lambda_1 t} + a_2 e^{-\lambda_2 t} = 0.90\, e^{-0.06t} + 0.10\, e^{-0.003t}$$

The two functions $0.90e^{-0.06t}$ and $0.10e^{-0.003t}$ are each represented by a straight line. The closeness of fit of the theoretical curve to the experimental data throughout the period of observation is apparent.

Such a representation would imply a system of two interconnecting pools rather than the single pool system previously hypothesized. In any multi-pool system of a given element or substance, the pools may be defined as aggregates of the substance which are distinct from each other either by virtue of spatial separation or because of differences in physiological or chemical properties.

Since such a 2-pool system approximates the experimental data within the limits of error, the consideration of more complex pool systems is unnecessary and mathematically unjustified under present conditions. This statement is not invalidated by the fact that preliminary observations on plasma iron disappearances followed for longer periods than the present 6-hour interval, have already indicated the necessity for the assumption of at least a 3-pool system. Although the approximating equations for such a system would have at least 3 exponential terms, the derived constants for the first 2 pools would differ from those obtained by the above 2-pool concept only within the limits of the present experimental errors.

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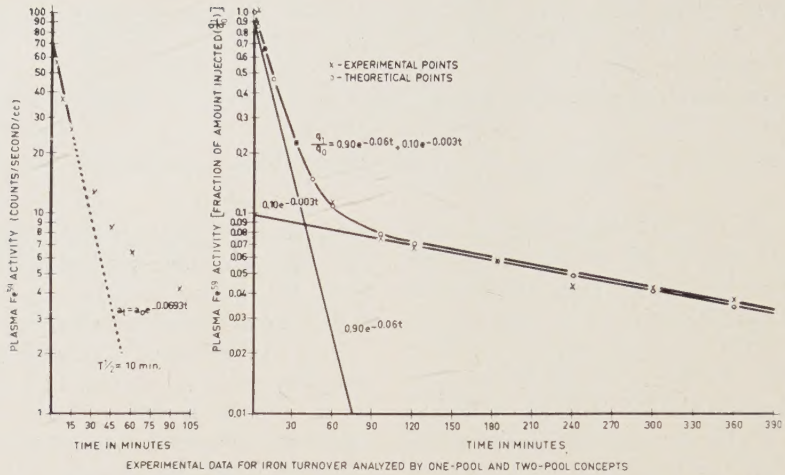

FIG. 1. A. Experimental data obtained during 1st hr of Fe^{59} disappearance from plasma following injection of ferric^{59} beta-1-globulinate in a case of polycythemia vera. Values for radioactivity are plotted against time on semi-log paper and may be approximated by a single exponential function. Note deviation from a straight line after 30 min. B. Experimental data of the same case plotted for a 6 hr period and approximated by the sum of 2 exponential functions. Note closeness of approximation.

Fig. 2 gives a schematic representation of the most general one- and 2-pool systems. Q_1 and Q_2 denote the pools themselves, as well as the quantity of stable iron comprising each of the pools. It is assumed that the pools empty at constant relative rates k_1 and k_2 respectively, *i.e.*, the amount k_1Q_1 (or k_2Q_2) leaves the pool Q_1 (or Q_2) per unit time. The relative rate is thus defined as a multiple or fraction of the absolute quantity in the given pool leaving it per unit time, in contrast to the conventional expression of rates in terms of absolute quantity per unit time. A constant fraction A_1 (or A_2) of the amount $k_1Q_1(k_2Q_2)$ that leaves pool $Q_1(Q_2)$ is eliminated entirely from the 2-pool system at an absolute rate $E_1 = A_1k_1Q_1(E_2 = A_2k_2Q_2)$. The remaining fraction $\bar{A}_1 = 1 - A_1(\bar{A}_2 = 1 - A_2)$ is transferred to the other pool at the absolute rate $\bar{A}_1k_1Q_1(\bar{A}_2k_2Q_2)$. The rates of supply of stable iron to the 2-pool system through Q_1 and Q_2 are denoted by S_1 and S_2, respectively.

The amounts of tracer iron associated with each of the stable pools at a given time are represented by q_1 and q_2 or more precisely, $q_1(t)$ and $q_2(t)$. At zero time ($t = 0$) a quantity $q_0 = q(0)$ of the tracer iron is introduced into Q_1. It is assumed that all mixing is instantaneous, and that therefore q_1 and q_2 are subject to the same constant relative rates as Q_1 and Q_2, respectively.

The amount of tracer remaining in Q_1 at time t can be given by general equation: $q_1 = q_0(a_1e^{-\lambda_1 t} + a_2e^{-\lambda_2 t})$ where a_1, a_2, λ_1, and λ_2, are functions of k_1, k_2, and $\bar{A}_1\bar{A}_2$:

$$a_1 = \frac{\sqrt{(k_1 + k_2)^2 - 4(1 - \bar{A}_1\bar{A}_2)k_1k_2} + (k_1 - k_2)}{2\sqrt{(k_1 + k_2)^2 - 4(1 - \bar{A}_1\bar{A}_2)k_1k_2}}$$

$$a_2 = \frac{\sqrt{(k_1 + k_2)^2 - 4(1 - \bar{A}_1\bar{A}_2)k_1k_2} - (k_1 - k_2)}{2\sqrt{(k_1 + k_2)^2 - 4(1 - \bar{A}_1\bar{A}_2)k_1k_2}}$$

$$\lambda_1 = \frac{(k_1 + k_2) + \sqrt{(k_1 + k_2)^2 - 4(1 - \bar{A}_1\bar{A}_2)k_1k_2}}{2}$$

$$\lambda_2 = \frac{(k_1 + k_2) - \sqrt{(k_1 + k_2)^2 - 4(1 - \bar{A}_1\bar{A}_2)k_1k_2}}{2}$$

Part (b) of Fig. 1 demonstrates the adequacy of the approximation of this equation to the actual data obtained in a case of polycythemia vera. The derivation of pertinent differential equations and of the above solution, as well as all subsequent mathematical treatment will be fully developed later(9).

While the values for k_1,k_2 and $\bar{A}_1\bar{A}_2$ can be obtained from the above equations, these con-

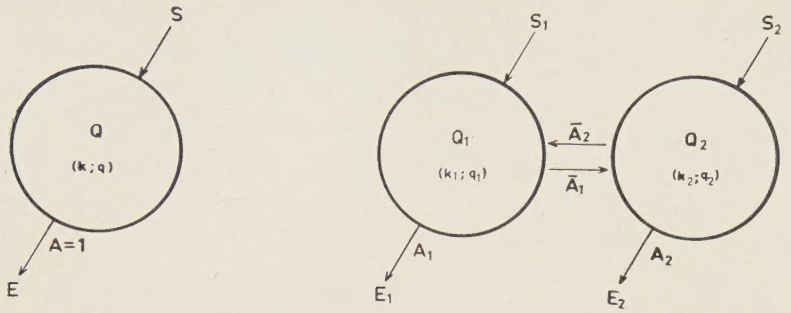

FIG. 2. A. Left: Schematic representation of a one-pool system. B. Right: Schematic representation of a 2-pool system. Symbols employed are defined and discussed in text.

stants have no immediate physiological significance. To derive meaningful parameters such as the amount of stable iron eliminated from the system through both pools per unit time which may be called the "effective" disappearance or turnover rate ($R_e = E_1 + E_2$), it must be assumed that the system is in a state of dynamic equilibrium, *i.e.*, the same amounts of stable iron enter as leave the system as a whole, as well as each of its pools separately. Such an equilibrium condition can be realized, however, in a variety of ways, by assuming different modes of entry and exit, of stable iron. On the basis of these a series of representative models can be constructed from which it is possible to obtain values for the "effective" disappearance rate as well as other parameters. Insofar as such values can be estimated by independent technics, it is possible to eliminate those models which are incompatible with known facts.

When a series of such 2-pool models is examined for the values of the effective disappearance rate, R_e, it can be divided into 2 distinct groups.

The first group of models gives either abnormally high or abnormally low hemoglobin renewal rates in all cases considered, including the normals. It seems reasonable therefore to exclude these models from further consideration.

The second group consists of models which in polycythemia vera yield values for R_e and thus for hemoglobin renewal rate, significantly lower than those obtained by the use of the single pool hypothesis and therefore more closely approximating normal red cell survival. In normal cases, on the other hand, and in most of the anemias, this group of models gives hemoglobin renewal rates essentially the same as those obtained by calculations using a single exponential function (Table I). The half-time of disappearance $T\frac{1}{2}$ has no direct meaning within the multiple pool concept and is not required in any of the calculations. For the majority of the models in this group $R_e = (1 - \bar{A}_1\bar{A}_2)\ k_1\ Q_1$, and therefore only this value is given in Table I.

Two of the cases of polycythemia vera (Cases I and II) showed progressive increase in red cell mass immediately subsequent to the experimental period. This implies a rate of hemoglobin synthesis in excess of the normal destruction rate of approximately 1% per day. These cases showed "effective" hemoglobin renewal rates which were moderately elevated (1.4% and 1.9%) yet significantly lower than the extremely high values (4.8% and 4.4%) obtained with the calculations based on the single-pool hypothesis.

Most of the models assume spatial separation of the pools with plasma iron as the first pool. It is theoretically possible that both iron pools are contained in the plasma yet differ from each other in chemical state or in reactivity.

The number of acceptable models can thus be reduced by considering the single parameter R_e. Although all the models in the second group give similar values for R_e, they differ widely in other constants. *In vivo* counting in humans may yield information as to the nature and location of the second pool Q_2, and further reduction in the number of acceptable models may follow. By such a

TABLE I. Comparative Data and Calculations on Iron Turnover Rates and Hemoglobin Renewal Using 1- and 2-Pool Systems.

Data	Case I Poly. vera	II Poly. vera	III Poly. vera	IV Sec. poly.	V Normal	VI Normal
Total plasma iron (Q_1) (mg)	1.70	1.20	4.85	3.21	5.18	4.12
Total RBC iron (mg)	3540	2280	3690	4150	2960	2260
Net RBC iron uptake (%)	100	91	99	93	84	100
1-pool model						
Parameter obtained from approximating curve:						
$T\frac{1}{2}$ (min.)	10	11	60	31	126	135
Derived parameter:						
Rate constant k (min.$^{-1}$)	.0693	.0630	.01155	.0224	.0055	.00513
Clinical parameters:						
Plasma iron turnover (mg/day)	170	109	80.7	103	41.0	29.7
RBC iron turnover (mg/day)	170	99.1	79.9	96.1	34.5	29.7
Hgb. renewal (%/day)	4.79	4.35	2.16	2.32	1.16	1.31
2-pool model (group II)						
Parameters obtained from approximating curve:						
λ_1 (min.$^{-1}$)	.06	.065	.013	.040	.020	.015
λ_2 (min.$^{-1}$)	.03	.045	.0025	.008	.0043	.003
A_1	.900	.896	.730	.530	.173	.340
A_2	.100	.104	.250	.470	.827	.660
Derived parameters:						
k_1 (min.$^{-1}$)	.054	.0587	.0102	.025	.0070	.0071
k_2 (min.$^{-1}$)	.0087	.0108	.0053	.023	.0173	.0109
$\bar{A}_1\bar{A}_2$	.620	.539	.400	.490	.306	.430
Clinical parameters:						
$R_e = (1-A_1A_2)k_1Q_1$ = effective plasma iron turnover (mg/min.)	.0349	.0325	.0297	.0449	.0252	.0163
R_e (mg/day)	50.2	46.8	42.7	64.7	36.2	23.4
Effective RBC iron turnover (mg/day)	50.2	42.6	42.3	60.2	30.4	23.4
Effective Hgb. renewal (%/day)	1.42	1.87	1.14	1.45	1.03	1.04

process of elimination a single acceptable model may eventually be arrived at which could be considered as a second approximation to the kinetics of iron metabolism.

In *summary* the 2-pool concept of iron kinetics appears to be a useful hypothesis giving a good approximation to the experimental data. Its application leads to rational values for hemoglobin renewal and red cell survival, particularly in polycythemia vera, thus obviating the need for more complex hypotheses such as double red cell populations in various blood dyscrasias(10).

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